ANALYSES OF MURINE BETA DEFENSINS AND DEFENSIN INSPIRED PEPTIDES

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Declaration

I declare that this thesis has been composed by me and that all of the work is my own, unless otherwise stated.

Karen Taylor

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List of abbreviations and symbols

°C	degrees centigrade
µCi	microcurie
µg	microgram
µl	microlitre
µM	micromole
aa	amino acid
Å	angstrom unit
A_{550}	absorbance
AMV	avian myeloblastosis virus
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
Ca^{2+}	calcium ion
CCL20	chemokine ligand 20
cDNA	complementary DNA
CD	circular dichroism
CFU	colony forming units
CM	chemotaxis media
CO_2	carbon dioxide
D_2O	deuterium oxide
DEPC	diethylpyrocarbonate
DMEM	Dulbeccos Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid
FCS	foetal calf serum

Fmoc	Fluoromethyloxycarbonyl
FMLP	formyl methionine leucine proline
FT-ICR	Fourier-transform ion cyclotron
g/l	grams/litre
HBSS	Hanks Balanced Salt Solution
HPLC	High Pressure Liquid Chromatography
HBD-	human beta defensin
HBTU	benzotriazole-tetramethyl-uronium hexafluorophosphate
HNP	human neutrophil peptide
HOBt	hydroxybenzotrizole
H_2O	water
Hprt	murine hypoxanthine phosphoribosyltransferase gene
IFNγ	interferon gamma
IL-	interleukin-
IU/ml	Internaltional Units per millilitre
IgG	immunoglobulin G
ISA	Iso-sensitest agar
ISB	Iso-sensitet broth
kb	kilobase
kDa	kiloDalton
l	litre
LPS	lipopolysaccharide
mM	millimolar
M	molar
MACS	Magnetic Cell Sorting
MALDI-TOF	Matrix-assisted laser desorption/ionisation time of flight
Mb	mega-base
MBC	Minimum Bactericidal Concentration
mbd	murine β-defensin peptide
mBD	murine β-defensin gene

MCP-1	monocyte chemoattractant proteins
MIP3α	Macrophage Inflammatory Protein 3 α
mg	milligram
mins	minutes
ml	millilitre
mM	millimolar
mRNA	messenger RNA
MRSA	Methicillin Resistant *S.aureus*
MS	mass spectrometry
NaCl	sodium chloride
nm	nanometer
NF-κB	Nuclear factor kappa B
ng	nanogram
NMR	Nuclear Magnetic Resonance
PBS	phosphate buffered saline
PCR	polymerase chain reaction
$p(dN)_6$	random 6 base pair primers
Q-ToF	Quadruple time of Flight
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse trsnscription
SD	standard deviation
SDF-1α	Stromal cell derived factor-1
SDS	Sodium Dodecyl Sulphate
SSC	sodium chloride/sodium citrate
TAP	bovine tracheal antimicrobial peptide
TBE	Tris, boric acid, EDTA buffer
TFA	Trifluoroacetic acid
TNF-α	tumour necrosis factor-α
Tm	melting temperature

TSB	tryptone soya broth
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

ABSTRACT

Antimicrobial peptides are important components of the innate immune systems ability to fight disease. They display widespread distribution throughout the animal and plant kingdom and directly inhibit infection rapidly. In numerous cases these peptides provide additional roles acting upon the immune system to co-ordinate a protective response. The defensins, one of the largest antimicrobial peptide families, can be subclassed into the α, β and θ defensins based on their six cysteine spacing and connectivity. The β-defensins which are expressed at a variety of epithelial surfaces, display antimicrobial properties and play important roles in host defence.

The aim of this project was 1) to evaluate the antimicrobial and chemoattractant properties of a novel five cysteine defensin related peptide (Defr1), 2) to analyse Defb14, the murine orthologue of human beta defensin 3 and 3) to investigate the properties of defensin inspired peptides.

This thesis describes the antimicrobial and chemoattractant properties of Defr1. This work suggests that Defr1 displayed antimicrobial activity against a panel of organisms for which antimicrobial treatment is limited or non-existent. In addition Defr1 displayed potent chemoattractant properties regarding various immune cell types and is the first example of a β-defensin that does not act through CCR6.

Data presented in this thesis describes the first study of the murine peptide Defb14. This concluded that a synthetic preparation of Defb14 displayed potent antimicrobial activity and roles within immune cell migration.

In this thesis, various defensin inspired peptides were subjected to study. Defb14 was used as a template for functional analysis and concluded that smaller N-terminal peptides displayed potent antimicrobial activity. Interestingly chemotactic activity was absent and toxicity remained similar to that of the original Defb14. These studies have highlighted the role of defensin inspired peptides as potential therapeutic agents.

CHAPTER 1:

INTRODUCTION

1.1 Requirement for novel antimicrobials

Since the discovery of penicillin, the management and eradication of bacterial infections has been revolutionised. The identification and development of the first lifesaving antimicrobial has encouraged further drug design to help combat a spectrum of micro-organisms. Currently an impressive range of antimicrobial agents are available providing treatment against an array of organisms. Unfortunately the end of the antimicrobial era is upon us with the decline in efficacy becoming a common occurrence. The misuse of these agents has resulted in a prevalence of multiple drug resistant organisms within the population.

The need for antimicrobial agents is frequently stated, with an increase in hospital acquired infections and highly resistant organisms, problems with unsuccessful treatment are common. New effective antimicrobial agents are not emerging to meet the demand as frequent as they did in the past. Pharmaceutical companies are reluctant to participate in the research and development of anti-infective agents. Within the past ten years the number of anti-infective programmes within large pharmaceutical companies has declined dramatically. Key players in drug discovery are abandoning the field in favour of long term chronic illnesses. These ailments require continued drug therapy and therefore a continued income for the company. The majority of antimicrobials prescribed cure the infection and the demand for long term therapy is minimal. Despite this the global market for antibiotics in 2002 was in the region of US $ 25 billion (Coates *et al.*, 2002). Although finance is a huge implication in development the task of developing novel antimicrobials is vast. New classes are required with novel targets and mechanisms. The antimicrobial pipeline is empty as the source of targets is small and very limited. In addition the cost of research for potential agents is not worth forsaking for the limited financial gain.

1.2 Antimicrobial peptides

Antimicrobial peptides are important components of the innate immune response common to invertebrates, vertebrates and plants. Throughout evolution these peptides have retained their antimicrobial potency and are thought to have arisen from multiple,

independent sources, possibly through convergent evolution (Zasloff *et al* 2002). These small diverse peptides provide a rapid first line of response against infection and are generally less than 10 kDa in size, contain 12-60 amino acids and have a cationic nature.

The majority of peptides can be grouped into three structural classes (Yount *et al.*, 2006), linear peptides often of α-helical tendancy, cysteine stabilised peptides mostly conforming to a β-sheet structure and those with one or more predominant amino acid residues, but variable in structure. The linear non- cysteine peptides such as magainin and cecropin are found within the skin secretions of amphibians and in insects respectively. While many of these peptides exist as extended or unstructured in aqueous solution, they have the ability to assume the α-helical structure within the membrane. The cysteine containing β-sheet peptides are the most prominent class of the antimicrobial peptides and consist of defensins and protegrins. These peptides are frequently comprised of several anti-parallel β-strands, stabilised by disulfide bonds which occasionally contain an α-helix. A common motif was discovered through proteomic analysis (Yount *et al.*, 2004) and termed the γ-core. This is composed of two anti-parallel β-sheets, with basic residues polarised along its axis. This structural motif is presenting all the major classes of cysteine stabilised host defense peptides. There are many examples of antimicrobial peptides in which one or more amino acid residues are dominant. For example, histatins contain predominant histidine residues and indolicidin contains increasing amounts of tryptophan.

Antimicrobial peptides are ideal candidates for potential theraputics in the field of anti-infectives. Considerable commercial and academic research has focused on developing a new generation of antimicrobial agents based on these natural antibiotics (Hancock *et al.*, 2002). Numerous companies have been established from antimicrobial peptides, Helix Biomedix, Novazymes, Novabiotics, Intrabiotics and Micrologix Biotech Inc with all having lead peptides in research programmes and in clinical trials.

1.3 The defensin family

The defensins are diverse members of a large family of antimicrobial peptides, widely distributed in plants, mammals and insects. These cysteine-rich peptides vary in their length, the spacing of their cysteine residues and their disulfide connectivities. The defensins are generally small (3-6kDa), cationic peptides and were originally isolated from rabbit neutrophil granules (Selsted *et al.*, 1985).

1.3.1 Classification of the defensins

The classification of defensins is based on the spacing and arrangement of their disulfide bridges (Figure 1.1) that stabilise the β-sheet structure. Mammalian defensins can be classified into three subfamilies; the originally isolated α-defensin (also termed classical defensins), the β-defensins and the more recently identified θ-defensins (Tang *et al.*, 1999). Insect and vertebrate defensins all contain six residues with variations in the spacing of the cysteine residues and their disulfide connectivities (reviewed in Yang *et al.*, 2001). Regarding insect defensins the cysteine residues are connected C1-C4, C2-C5 and C3- C6. The connectivities of the α-defensins are C1-C6, C2-C4 and C3-C5 and the β-defensins are reported to have C1-C5, C2-C4 and C3-C6. The θ-defensins (with the rhesus θ-defensin 1) display the C1-C6, C2-C5 and C3-C6 connectivities. Defensins are also found in some plant species (reviewed in Broekaert *et al.*, 1995) but these display eight cysteine residues and are linked C1-C8, C2-C5, C3-C6 and C4-C7.

1.3.2 The mammalian defensin subfamilies

The mammalian defensin gene family are present at five syntenic loci in mouse and human genomes with the main locus on human chromosome 8p22-23 and mouse chromosome 8A3 (Schutte *et al.*, 2002). The different subfamilies of defensins are thought to share a common ancestry (Linzmeier *et al.*, 1993) despite their evolved differences.

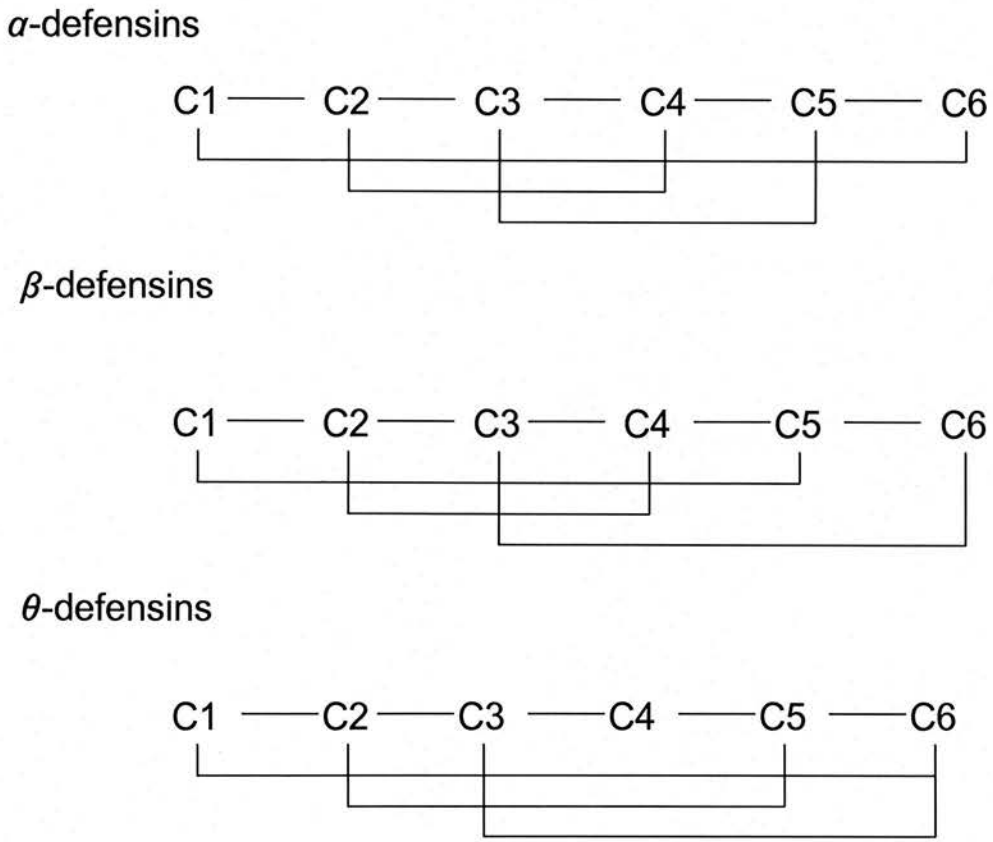


Figure 1.1 Cysteine connectivity of the defensins

The classification of defensins is based on the spacing and arrangement of their disulfide bridges. The connectivities of the α-defensins are C1-C6, C2-C4 & C3-C5. The β-defensins are C1-C5, C2-C4 & C3-C6. The θ-defensins display C1-C6, C2-C5 &C3-C6.

1.3.2.1 The α-defensin subfamily

The α-defensins were initially identified in the granules of both rabbit and human neutrophils (Selsted *et al.*, 1985; Ganz *et al.*, 1985). These defensins (HNP-1-4) are abundant in granules of polymorphonuclear leukocytes (PMN) or expressed by paneth cells within the human gut (HD-5, HD-6) (Selsted *et al.*, 1992). Mouse neutrophils do not express detectable amounts of α-defensins (Eisenhauer *et al.*, 1992) but they do express many enteric α-defensin peptides known as cryptidins in intestinal paneth cells (Ganz *et al.*, 2003). The α-defensins are encoded by genes comprising three exons and are initially synthesised as prepropeptides (Lehrer *et al.*, 1993). The prepropeptides contain an endoplasmic reticulum signal sequence of approximately 20 residues, a 45 amino acid residue anionic propiece and a mature cationic peptide of around 30 residues. The signal sequence is cotranslationally removed from its amino terminus and the mature peptide is generated. Cleavage of the propiece has been shown to be required before antimicrobial activity can be observed (Valore *et al.*, 1996). The HNPs are synthesised by promyelocytes and the mature peptides are stored in the primary granules of neutrophils (Ganz *et al.*, 2003). Unlike the human neutrophil peptides, HD-5 is released as a propeptide and is processed extracellularly and is mediated with trypsin (Ghosh *et al.*, 2002). Regarding the mouse cryptidins, cleavage of the propiece occurs extracellularly and is mediated using matrilysin (Wilson *et al.*, 1999)

The human neutrophil peptides predominantly have a β-sheet structure stabilised by the presence of their three disulfide bonds. NMR studies have been performed on various human neutrophil peptides. HNP-3 has been crystallised to 1.9 Å resolution (Hill *et al.*, 1991) revealing a dimeric structure.

The mature α-defensins have been shown to possess broad spectrum antimicrobial activity against both Gram positive and negative organisms. *S. aureus*, *P. aeruginosa*, *E. coli*, *C. neoformans* have exhibited varying antimicrobial action (Miyasaki *et al.*, 1990). HNP-1 and HNP-2 have shown activity with *C. albicans* (Lehrer et al., 1988) but HNP-3 was inactive. The antimicrobial activity has also been shown to diminish in the presence of a high salt environment (Lehrer *et al.*, 1988). Not

only do the α-defensins display antimicrobial activity, evidence has emerged that they have potent antiviral activity (Daher *et al.*, 1986). Herpes simplex virus, cytomegalovirus, influenza and HIV have been shown to be inhibited (Klotman *et al.*, 2006)

Human neutrophil peptides have been shown to possess an array of additional activities (Yang *et al.*, 2001). The α-defensins from humans, rabbits and guinea pigs have been shown to induce histamine release and peritoneal mast cell degranulation (Befus *et al.*, 1999). Human α-defensins have been shown to interact with complement, binding to C1q (Panyutich *et al.*, 1994). Chemotaxis has been observed with the migration of immature dendritic cells and naïve T-cells (Yang *et al.*, 2000).

1.3.2.2 The θ-defensin subfamily

The θ-defensins are the most recent defensin family to be identified from Rhesus macaque leukocytes. Rhesus theta defensin-1 (RTD-1) is formed from two different α-defensin precursors involving the head to tail ligation and the formation of two new peptide bonds (Tang *et al.*, 1999). RTD1a and RTD1b are translated, their ends are spliced and both are ligated together producing a circular defensin (Figure 1.2). The cyclic peptide contains 18 residues and forms 3 disulfide bonds. Two homodimeric θ-defensins (Tran *et al.*, 2002) RTD-2 and RTD-3 are predicted splicing products of RTD-1a and RTD1b and were purified from rhesus monkey bone marrow. Genes encoding functional peptides have only been found in Rhesus monkeys. Human bone marrow expresses a pseudogene that encodes antimicrobial peptide homologoues to the circular rhesus monkey mini-defensins (θ-defensins) termed retrocyclin (Cole et al., 2002). Retrocyclin was made synthetically and was shown to protect human CD4+ cells from infection by HIV-1 in vitro, was noncytotoxic and demonstrated effective killing against certain organisms. In the human genome at least six genes for coding the θ-defensins have been found. Premature stop codons in the genes abort translation and subsequent peptide production (Selsted *et al.*, 2004, Pazgier *et al.*, 2006).

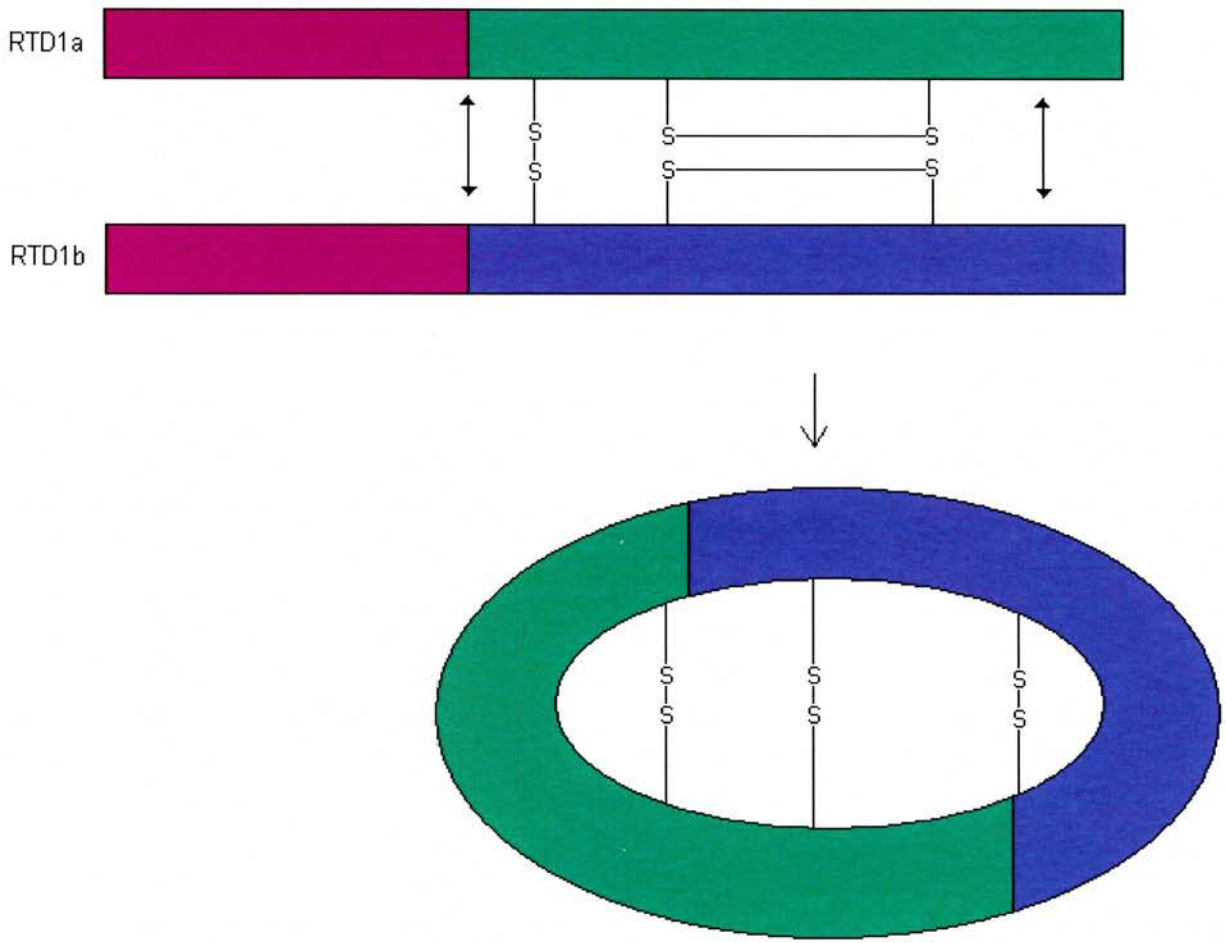


Figure 1.2 Posttranslational processing of RTD-1 precursors
Adapted from Tang *et al*., 1999
Small arrows indicate where RTD1a and RTD1b are spliced, generating two nonapeptides and then ligated to produce the circular mature RTD1

1.3.2.3 The β-defensin subfamily

The β-defensins are produced by vertebrates including mammals and birds. The first β-defensin reported was isolated from the tongue of a cow and termed tracheal antimicrobial peptide (Diamond *et al.*, 1992). β-defensins were subsequently found in bovine granulocytes (bovine neutrophil β-defensins, BNBD1-13 (Selsted *et al.*, 1993)), and in chicken leucocytes (gallinacin, Gal-1α and Gal1-2 (Harwig *et al.*, 1994)). The spacing and connectivity of the cysteine residues (C1-C5, C2-C4 and C3-C6) differed from those observed with that of the α-defensins (C1-C6, C2-C4 and C3-C5).

1.4 Human β-defensins

Numerous human β-defensin genes exist but four human β-defensins have been described in great detail (HBD1-4) although at least 28 more have been reported (Schutte *et al.*, 2002). Human β-defensin 1 was the first human defensin identified and purified from the plasma of renal patients (Bensch *et al.*, 1995). Human β-defensin 2 was originally isolated from psoriatic skin lesions (Harder *et al.*, 1997) similar to human β-defensin 3 which was purified from psoriatic scales (Harder *et al.*, 2001). Human β-defensin 4 was originally reported through genomic analysis (Garcia *et al.*, 2001a).

HBD1 was identified from human blood filtrate as a 36 amino acid mature peptide with sequence homology to that of the bovine defensins (Bensch *et al.*, 1995). HBD1 is constitutively expressed in a variety of tissues including the pancreas and kidney with lower levels detected in the salivary glands, testis, thymus, placenta and small intestine (Zhao *et al.*, 1996). HBD1 expression was also detected in the collecting ducts and the loops of Henle (Schnapp *et al.*, 1998). The HBD1 gene (DEFB1) spans a 7 kb region with an intron of 6962 bp separating two exons (Liu *et al.*, 1997). HBD1 is not inducucible by inflammatory stimuli of bacterial products including lipopolysaccharide (LPS), interleukin 6 (IL-6), Interferon- γ (IFN-γ), phorbol myristate aetate (PMA) or tumor necrosis factor- α (TNF-α) (Zhao *et al.*, 1996). The promoter region does not contain the necessary transcription factor binding sites required to respond to these factors.

HBD2 was isolated from lesional psoriatic scale extracts as a 41 residue peptide (Harder *et al.*,1997). Human β-defensin 2 is the first member of the family found to be an inducible peptide. Expression of this peptide has been detected in the skin, lung, trachea, urogenital tract, salivary gland and uterus (Harder *et al.*, 1997; Bals *et al.*, 1998a; Hiratsuka *et al.*, 1998). HBD2 is induced in response to microbial and host factors due to the presence of one or more multiple nuclear factor kappa B (NF-κB) consensus binding sites. The flanking region contains four NF-κB sites that are not evident in DEFB1 gene but are in TAP, which is also upregulated by inflammatory stimuli (Russell *et al.*, 1996). Recognition of bacterial products and proinflammatory cytokines resulted in NF-κB activation and transcription of HBD-2 (Becker et al., 2000; Diamond et al., 2000). HBD2 expression has also been shown to be mediated by Toll Like Receptor -2 (TLR2) (Birchler *et al.*, 2001). The peptide has also been shown to act like an endogenous ligand for TLR 4 on immature dendritic cells and induced dendritic cell maturation (Biragyn *et al.*, 2002).

The third human defensin to be identified was human β-defensin 3 (HBD3) and similar to HBD2 this was purified from psoriatic skin lesions (Harder *et al.*, 2001).
The search for the third defensin was driven by the fact that the previous peptides could not kill gram positive organisms within lesional keratinocytes. It was hypothesised that additional peptides must be present displaying activity against *S. aureus*. HBD3 can be found to contain 45 amino acid residues and is expressed skin, tonsils, gingival keratinocytes, skeletal muscle, adult heart, testis and esophagus (Harder *et al.*, 2001) and can kill *S. aureus*.

The fourth peptide to be identified was human β-defensin 4 and this was detected by genomic analysis (Garcia *et al.*, 2001a). HBD4 transcripts were detected in the testis at high levels while lower levels were present in the kidney, lungs, thyroid, stomach and uterus. The expression of HBD4 was found to be upregulated by exposure to bacteria and also to phorbol-12-myristate-13-acetate (PMA). Analysis of the flanking region displayed the presence of several AP-1 sites and GATA1 binding sites but no NF-κB or STAT binding sites (Garcia *et al.*, 2001b). HBD4 has been shown to be induced in primary respiratory epithelial cells by phorbol ester or *P. aeruginosa* or

S. pneumoniae. IL-1α, IL-6, IFN-γ and TNF-α do not induce HBD4 (Garcia *et al.*, 2001b).

1.5 Murine β-defensins

The first murine β-defensin to be identified, *Defb1* (*m*BD-1) was found based on the homology of the human β-defensin 1 (Huttner *et al.*, 1997; Morrison *et al.*, 1998). The *Defb1* gene contains two exons, the first encoding the signal sequence and part of the proregion, whereas the second exon encodes the remainder of the propiece and the mature peptide. Defb1 has been detected at high levels in the kidney, and also found at lower levels in the heart, lungs, gut and skeletal muscle. Defb1 has demonstrated salt sensitive antimicrobial activity against *E .coli*, *P. aeruginosa* and *S. aureus* (Morrison *et al.*, 1998; Bals *et al.*, 1998b). However when tested against *B. cenocepacia* (Morrison et al., 1998) the peptide failed to display antimicrobial activity. Mouse models deficient in Defb1 displayed higher numbers of *S. aureus* in the bladder (Morrison *et al.*, 2002a; Moser *et al.*, 2002).

The second murine β-defensin *Defb2* has also been reported (Morrison *et al.*, 1999). *Defb2* gene encodes a 71 amino acid peptide. The expression of Defb2 was detected in the lungs, skin, kidney, uterus and trachea and was found to be induced following treatment with lipopolysaccharide (LPS) unlike that of Defb1. Defb2 has been shown to bind to CCR6 and chemoattract immature dendritic cells but not mature dendritic cells (Biragyn *et al.*, 2001).

The murine β-defensin 3 was isolated from lungs using primer sequences based on a rat β-defensin (Bals *et al.*, 1999). Further analysis of the gene revealed the presence of a NF-κB site in the promoter region of the gene and a 1.7 kb intron separating two exons. Defb3 also contains an additional amino acid residue between the second and third cysteine residues and displays expression in the salivary glands, epididymis, liver, brain, ovaries and lung. Within the liver the expression was induced following exposure to *P.aeruginosa* via airway instillations.

Murine β-defensin 4 has also been reported (Jia *et al.*, 2000) with expression being detected in the oesophagus, trachea and tongue but not induced via inflammatory

stimuli. The gene comprises of two exons separated by an intron of 2.4 kb. Functional analysis data on this peptide has not been published.

1.6 Evolution of the defensins

The defensin subfamilies share common characteristics from their cationic properties, small size and cysteine residues. Structural and functional similarities suggest a common ancestor. Based on these observations it has been proposed that they possibly descended from a common ancestral gene by duplication and divergence (Hughes *et al.*, 1999). However it is difficult to determine whether the mammalian α-defensins, the vertebrate β-defensins and the insect defensins are homologous or have independently evolved. The spacing of the cysteine residues in the disulfide bonds and the three dimensional structure are evidence for a distant relationship. Nucleotide substitutions in recently duplicated mammalian defensin genes show that the rate of nonsynonymous substitution exceeds that of the synonymous substitution in the region of the gene encoding the mature peptide. This is evidence of positive selection driving the diversification of the defensins. The 8p22-p23 locus has evolved by successive rounds of duplication followed by substantial divergence involving positive selection (Semple *et al.*, 2003). Positive selection, disproportionately favours alterations in the charge of the amino acids and has been implicated as driving second exon divergence in these genes (Semple *et al.*, 2003).

1.7 Structure of the β-defensins

The structures of HBD1, HBD2 and HBD3 (Hoover *et al.*, 2000; Harder *et al.*, 2001; Schibli *et al.*, 2002) have been determined (Figure 1.3). Mouse β-defensin 7 and 8 have also been reported (Bauer *et al.*, 2001). The tertiary structures of these defensins are similar despite the differences in their amino acid sequences. The defensins consists of three β-strands arranged in an antiparallel sheet and held together by three intramolecular disulfide bonds. The β-sheet is flanked by an α-helical segment formed by the N-terminal fragment of the molecule (Pazgier *et al.*, 2006) although this is not present in the α-defensins and bovine β-defensins (Hill *et al.*, 1991). This α-helix

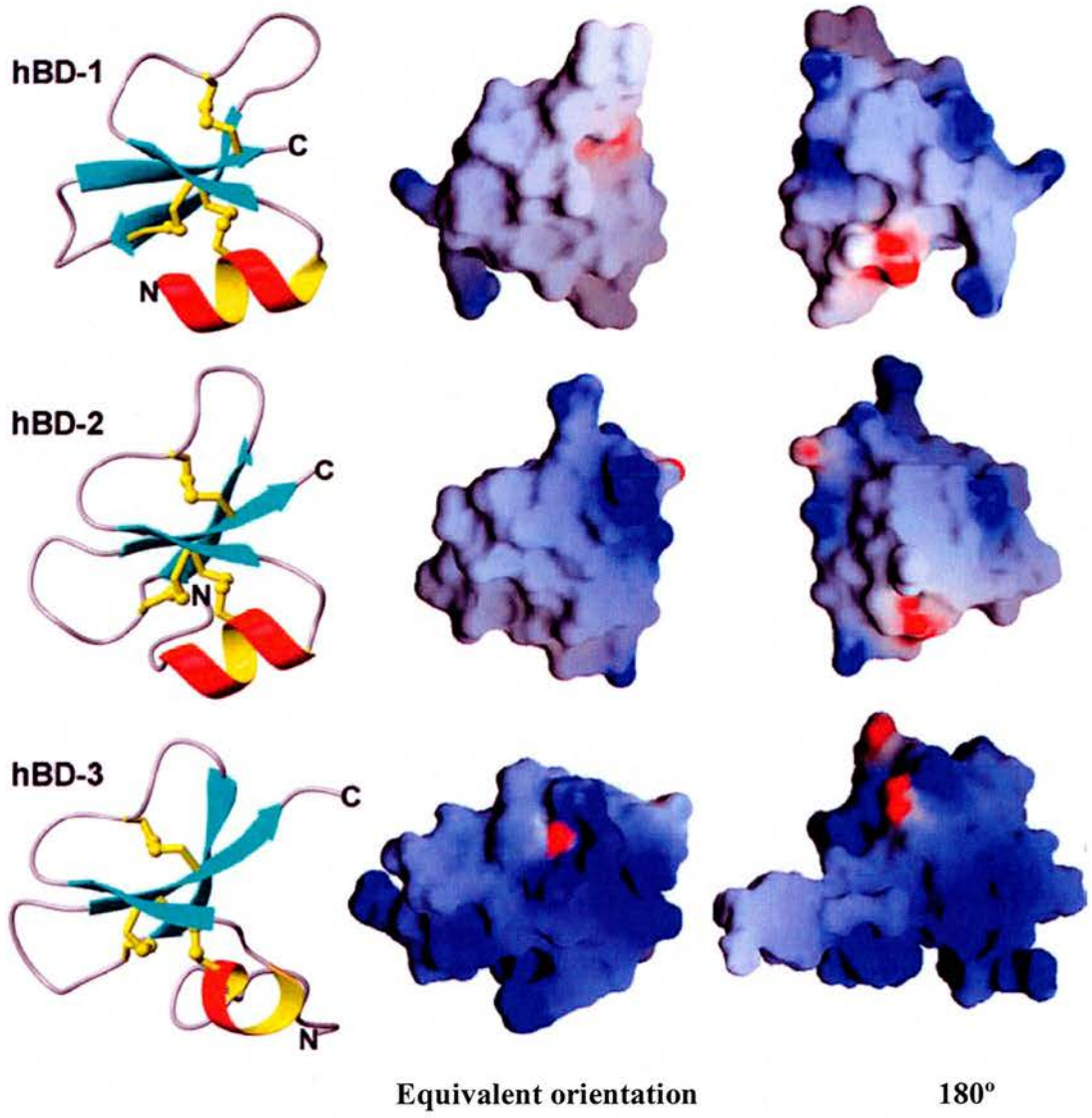


Figure 1.3 Structural comparisons of human β-defensin 1, 2 & 3

Schematic representation of HBD1, 2 and 3 displaying an N-terminal α-helix, 3 β-strands arranged in an antiparallel sheet and held together by three intramolecular disulfide bonds. The positively and negatively charged areas of the solvent-accesible surfaces are coloured in blue and red respectively. Figures were taken from Pazgier *et al.*, 2006, constructed using MOLMOL and Grasp.

orientation is stabilised by the disulfide bond (C1-C5) to the β-sheet. A conserved motif Gly-X-Cys is found in the second β-strand and forms a β-bulge (Hoover *et al.*, 2000). The β-bulge is thought to be responsible for a twist in the β-sheets and is necessary for the formation of the structure and the correct folding (Xie *et al.*, 2005). The residues forming the β-sheet have been shown to be less susceptible to negative or positive selection during the evolution of the mammalian β-defensin genes (Semple *et al.*, 2003).

1.8 Function of the β-defensins

The β-defensins display a diverse array of biological activities and possibly the most recognised is their ability to act as antimicrobials. Additional roles highlight their potential as immunomodulators indicating their potential roles in regulating both the innate and adaptive immune responses.

1.8.1 Antimicrobial action

The β-defensins have been branded initially as antimicrobial peptides and numerous studies have focused on these properties. The β-defensins have the capacity to kill or inhibit *in vitro* a wide variety of bacteria and fungi particularly at low concentrations of salt and plasma proteins (Selsted *et al.*, 1993; Ganz *et al.*, 1998). HBD1 and HBD2 have displayed effective antimicrobial properties against Gram-negative organisms but appear to be less potent against Gram positive organisms. HBD3 displays potent broad spectrum activity against both Gram positive and negative organisms (Goldman *et al.*, 1997; Bals *et al.*, 1999). The activity of the β-defensins has been shown to be inhibited by the presence of divalent cations (Tomita *et al.*, 2000) and sodium chloride (Garcia et al., 2001b).

The physicochemical properties of the β-defensins determine their antimicrobial activity and their specificity. The balance of net positive charge, the hydrophobic residues and the amphiphilic character determine the activity. All β-defensins share a very similar structural core, consisting of three β-strands arranged in an antiparallel sheet and held together by three intramolecular disulfide bonds. Microbial specificity

has been suggested to result from variable arrangements of the non-conserved residues (Semple *et al.*, 2003; Sahl *et al.*, 2005).

Knockout studies have provided some interesting results. Knockouts of a single defensin, mouse β-defensin-1, results in a delayed clearance of *Haemophilus influenzae* from the lung (Moser *et al.*, 2002) and increases colonization by *Staphylococcus* species in the bladder (Morrison *et al.*, 2002a).

1.8.1.1 Mechanism of action

Numerous studies have focused on the mechanisms responsible for antimicrobial action of the β-defensins and are as yet, not clear. The role of interactions has been extensively studied between negatively charged membranes and cationic peptides using artificial membranes (Bohling *et al.*, 2006; Wimley *et al.*, 1994). The defensins have been described as being membrane acting agents (Hancock *et al.*, 1998) targeting the cell membrane which also forms the basis of their selectivity. The positively charged peptides interact with the negatively charged structures of the microbial membranes. These components include teichoic acid in the Gram positive organisms and lipopolysaccharide in the Gram negative organisms. These structures are specific to prokaryotic cells and not found in eukaryotic cells therefore providing specificity for the prokaryotic membrane. In addition the membranes of the eukaryotic cells contain cholesterol and zwitterionic phospholipids that display no overall charge, althought the inner leaf display negatively charged properties.

The molecular details of the interactions between the β-defensins and the membranes are not clearly understood. Different models have been proposed and the precise mechanism remains to be investigated.

The initial attraction between the target cell and the peptide is electrostatic involving the positively charged peptide and the negatively charged cell membrane. The importance of this reaction has been confirmed experimentally for several of the α-defensins (Satchell *et al.,* 2003). Regarding Gram negative organisms, the peptides must cross the outer membrane first. Gram negative organisms consist of two cell envelope membranes, a cytoplasmic membrane and an outer membrane, separated by

periplasm containing a thin layer of peptidoglycan. The initial uptake within the Gram negative organisms is proposed to utilize the self-promoted uptake pathway (Hancock *et al.*, 2000). This mechanism was first proposed to explain the uptake of polycationic antimicrobials such as the aminoglycosides and polymyxins (Sawyer *et al.*, 1988). The peptides interact with the polyanionic surface of the membrane and have an affinity for LPS where they competitively displace the divalent cations Ca^{2+} and Mg^{2+}. The peptides disrupt the outer membrane, which in turn allows the uptake of the peptides into the periplasmic space (Hancock *et al.*, 1988).

Two different models have been proposed to describe the structure of the peptide membrane pore (Ganz., 1999). According to one model, the barrel stave model (Figure 1.4), the β-defensins might form channel-like pores spanning across the membranes. The peptides oligomerise like the staves of the barrel with the complex behaving like a transmembrane protein. This has been shown to be initiated with the peptide alamethicin (Brogden *et al.*, 2005) whereby the hydrophobic regions align with the lipid core region of the bilayer and the hydrophilic peptide regions form the interior region of the pore.

The second model, the carpet model (Figure 1.5), peptides accumulate on the surface bilayer. Peptides are electrostatically attracted to the phopholipid head groups at numerous sites covering the surface like a carpet. This neutralises the negatively charged lipids and the integrity of the bilayer is disrupted allowing passage of ions and larger molecules. This mechanism has been explained for ovispirin (Brogden *et al.*, 2005) which orientate parallel to the membrane surface.

Both the barrel stave model and the carpet theory have their advantages and disadvantages. The β-defensins ability to oligomerise and display an amphiphilic character (Pazgier *et al.*, 2006)) supports the barrel stave model although specific oligomerisation is required for forming the channel and this has not been directly observed with the defensins. According to the carpet model, this does not require extensive hydrophobicity or specific oligomerization. The hydrophobic regions of the peptides play a role in the stabilisation of the oligomeric structure and the charge dependant activity of these peptides agrees with the electrostatic interactions required

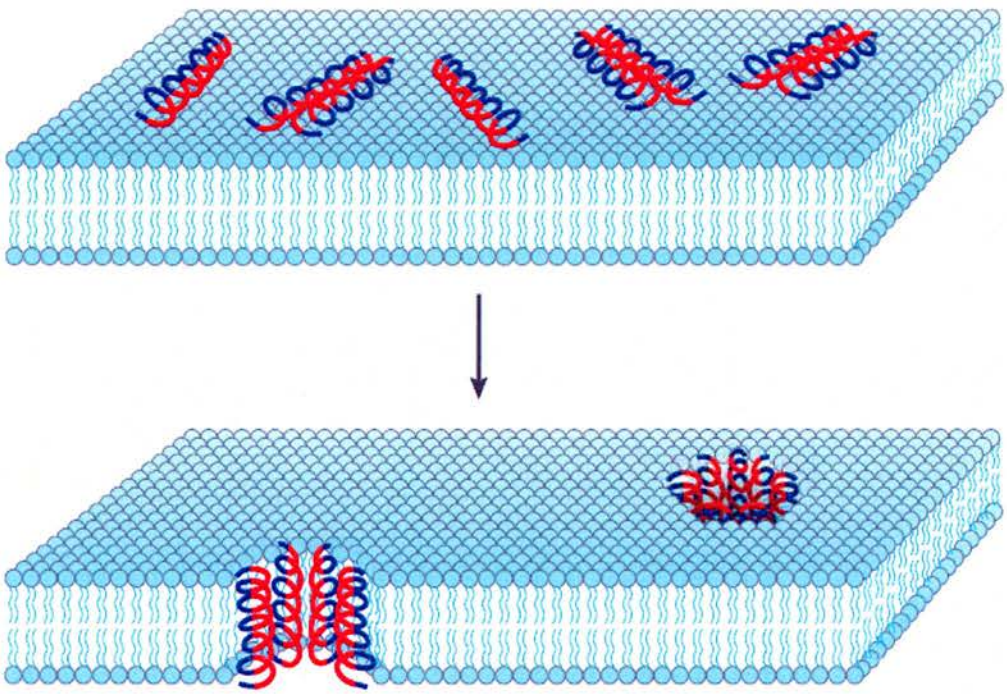


Figure 1.4 The barrel-stave model of antimicrobial peptide induced killing

The attached peptides aggregate and insert into the membrane bilayer so that the hydrophobic peptide regions align with the lipid core region and the hydrophilic peptide regions form the interior region of the pore. Hydrophilic regions of the peptide are coloured red and hydrophobic regions blue.
Figure taken from Brogden *et al.*, 2005

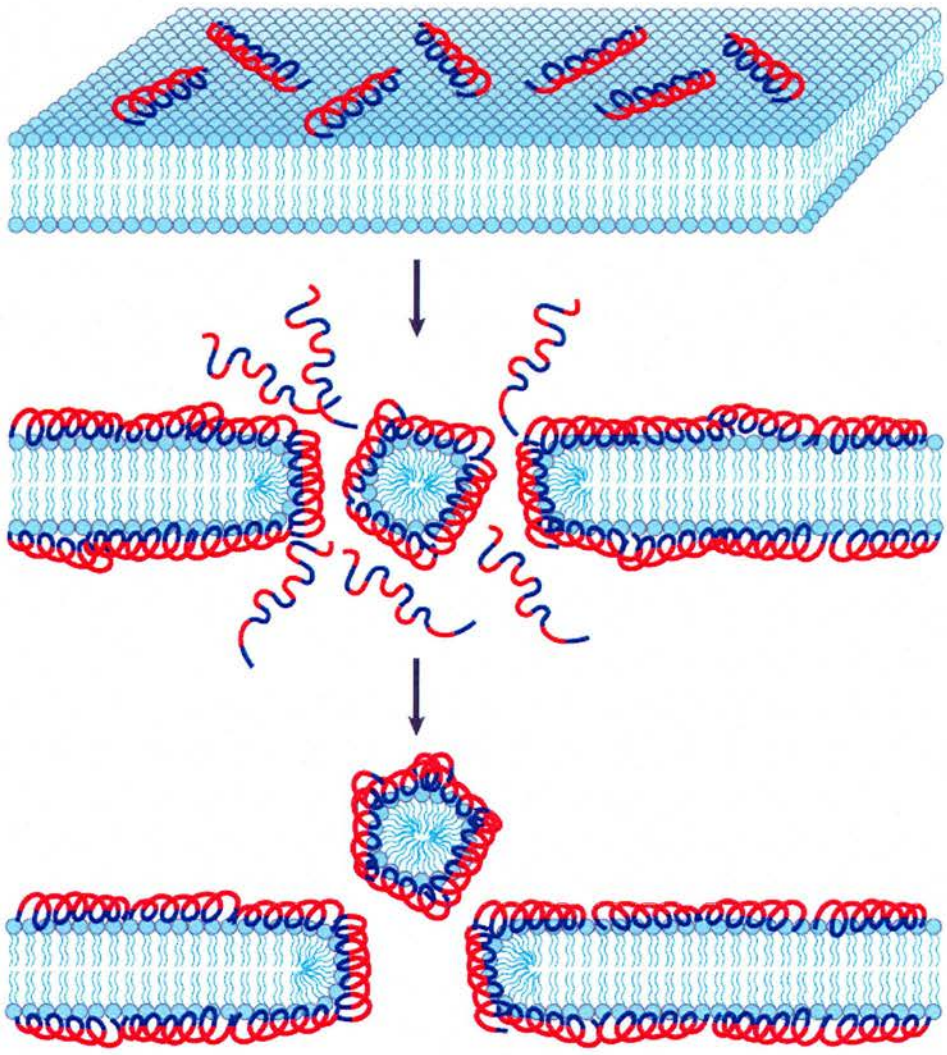


Figure 1.5 The carpet model of antimicrobial peptide induced killing

In this model the peptides orientate parallel to the surface of the lipid bilayer and forming an extensive layer or carpet. Hydrophilic regions are coloures red and hydrophobic regions of the peptide are coloured blue.
Figure taken from Brogden *et al.*, 2005

for the carpet model. Both the barrel stave model and the carpet model do not describe the full picture required for peptide membrane interaction. Further investigation is required to elucidate the mechanisms and the components involved in the killing.

How the peptide actually kills the target cell is subject to much debate. One theory suggests that the formation of channels in the cytoplasm results in loss of cell viability therefore being responsible for death (Falla *et al.*, 1996). Another theory implies that antimicrobial peptides enter the cell through the disrupted membranes and interact with intracellular targets (Hancock *et al.*, 1997; Brogden *et al.*, 2005).

1.8.1.2 Resistance mechanisms to antimicrobial peptides

Some species of bacteria have developed mechanisms to alter the structures involved in the interactions with cationic antimicrobial peptides. By altering these points the interaction with cationic antimicrobial peptides is diminished and subsequently the peptides become ineffective at killing. (Peschel *et al.*, 2001). Numerous organisms have developed resistance mechanism in hope of encouraging an established persistent infection. Mechanisms of resistance include membrane bound proteases, efflux pumps and modulation of outer membrane permeability through various structural modifications (Tzeng *et al.*, 2005)

The cystic fibrosis lung pathogen, *Burkholderia cenocepacia* is highly resistant to the action of antimicrobial peptides (Hancock *et al.*, 1997). Antimicrobial peptides like polymyxin have an inability to disrupt and permeabilise the outer membrane possibly connected to the external structures of LPS. *Burkholderia* LPS contain one third of the phosphate content present in the species *Pseudomonas* therefore limiting the potential cationic binding sites (Moore *et al.*, 1986). Recently it has been demonstrated that *B.cenocepacia* has to possess the complete core oligosaccharide attached to lipid A for it to maintain its resistance to antimicrobial peptides (Loutet *et al.*, 2006) (Figure 1.6). Furthermore, the LPS of Burkholderia has a change in the inner core. The Ko residue in the Ko-α(2-4)-Kdo disaccharide is substituted with Ara4N at position 8 (Gronow et al., 2003) although this isolate ATCC 25416 belongs to genomovar I which is not highly prevalent in cystic fibrosis infections.

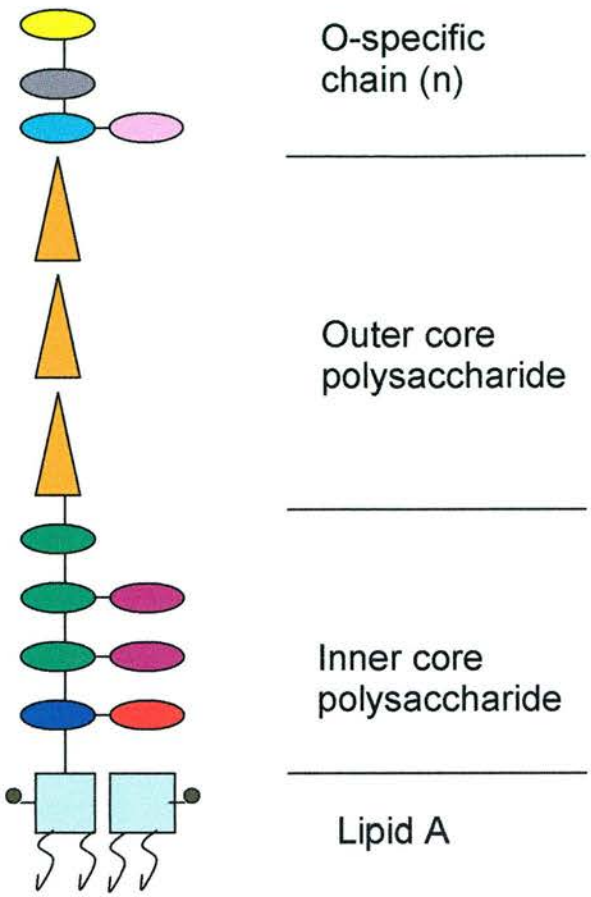


Figure 1.6 Burkholderia LPS Schematic

Structure of Burkholderia cepacia complex lipopolysaccharide (adapted from *Mahenthiralingam* et al 2005) displaying lipid A composed of two glucosamine residues with fatty acid side chains and phosphodiester-linked 4-amino-4-deoyarabinose. The inner core polysaccharide consists of 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) which is attached to lipid A and D-glycero-D-talo-oct-2-ulosonic acid (KO). In addition heptose moieties with sugars complete the inner core. Attached to the inner core are various hexose sugars which form the outer core region. The O-antigen is a repeating unit (n) of specific sugar residues that form the basis of serotyping.

Staphylococcus aureus has been reported to be resistant to a variety of antimicrobial peptides (Peschel *et al.*, 1999). The *mprF* gene of *S. aureus* determines resistance (Peschel *et al.*, 2001). *S.aureus* achieves resistance to cationic antimicrobial peptides by modifying anionic phosphatidylglycerol with L-lysine resulting in a repulsion of the positively charged peptides (Staubitz *et al.*, 2004).

1.8.2 Chemotaxis

Chemotaxis is defined as a process of direct migraton of cells along a chemical gradient. Within the immune system chemotaxis maintains a huge role in play with large numbers of immune related cells locating to sites of inflammation and infection. Many immune related cells possess the ability for chemotaxis (Luster *et al.*, 2002). A large number of chemoattractant substances have been identified, such as the chemokines. Chemokines are low molecular weight chemoattractants activating and inducing a variety of cell types (Murdoch *et al.*, 2000)

Many immune cells patrol throughout the body and migrate to a specific point following a chemotactic gradient (Delves et al., 2000). Using selectin molecules expressed on the surface of neutrophils and T-cells, inflammatory signals upregulate adhesion molecules such as Sialyl-Lewis^x on the surface of the vascular endothelium adjacent to the site of infection. The L-selectins on the immune cell surface interact with the adhesion molecules allowing the patrolling cell to bind to the endothelium adjacent to the site of infection. The immune cell sheds the L-selectin, integrins are activated and on the endothelium cells up regulate the E-selectins. The integrin interaction with the E-selectin arrest the rolling cell and allows firm adhesion to the endothelial surface. The immune cell then passes through the endothelial wall, moving up the chemotactic gradient towards the site where it is required.

Various chemokines attract the immune cell through the endothelium. These chemokines are immobilised on the endothelial surface via presentation molecules such as proteoglycan and also due to the production of chemokines by the endothelial cells themselves (Witko-Sarset *et al.*, 2000). The cell is suggested to be pulled through the

endothelium by the interaction of the immune cells integrins and the P-selectins concentrated on the endothelial cells (Burns *et al.*, 2000). The ability of the leuckocytes to navigate throughout the gradients of chemoattractants has been suggested by responding to one agonist source then another. The initial direction of the cell is dictated by the closest steepest chemoattractant gradient. This is then regulated by desensitisation of receptors, attraction by distant agonists and then end-target attractants such as formyl peptides and C5a. The chemotactic cells are elongated in shape and thought to be more sensitive at one end to an attractant. G-protein coupled receptors detect chemokines (Ijima *et al.*, 2002). The mechanism by which the cell senses the direction of the gradient remains unknown however it is proposed that the balance between the rapid excitatory and the slower inhibitory signals controlled by receptor binding is involved (Parent *et al.*, 1999). When the receptor binds to the chemoattractant, the excitation signal increases greatly and a response is generated until the inhibition signals reach the same level. If the excitation signal at the front of the cell exceeds the inhibitory signal at the back this generates a persistent excitation signal with the back of the cell the inhibition signal predominates and there is no response. Within this model, the duration of the response is related to the cells position within the gradient. The signal results in polymerisation of the cells F-actin at the site closest to the chemoattractant, leading to the formation of a new pseudopod, cell polarisation and a protrusion at the leading edge. This is followed by the assembly of conventional nonmuscle myosin and retraction of the posterior of the cell (Parent *et al.*, 1999).

The β-defensins have displayed chemoattractant properties with a variety of cell types. In order to elicit a coordinated response against invading pathogens specific cell types have to be recruited to sights of bacterial entry. Leukocyte recruitment occurs along gradients of chemotactic factors and is evident at nanomolar concentrations (Baggiolini *et al.*, 1998). Initially HBD1 and HBD2 were observed to recruit both human immature dendritic cells and memory peripheral blood memory T cells (Yang *et al.*, 1999). Additional studies involving the β-defensins showed that chemotaxis was evident with various defensins. HBD3 and HBD4 are shown to be chemoattractant for monocytes/macrophages (Garcia *et al.*, 2001a, Garcia *et al.*, 2001b). Activity with mast

cells has also been observed. HBD-2 has been shown to activate rat mast cells and in addition promotes the release of histamine and prostaglansin D_2 (Niyonsaba *et al.*, 2001).

The β-defensins have been shown to use chemokine receptor 6 (CCR6) (Yang *et al.*, 1999) which is also the receptor for Macrophage Inflammatory Protein 3 alpha (MIP-3α)/CCL20. The β-defensins have also been shown to be displaced from the CCR6 transfected cells by the chemokine MIP-3α suggesting that both have similar motifs.

The molecular details behind the receptor mediated chemotaxis of the β-defensins are unknown. The inclusion of the cysteine residues is thought to be important for chemoattractant properties. Using HEK293 cells expressing the human CCR6 cells Wu *et al.*, 2003 demonstrate that disulfide deficient HBD-3 displays no chemoattractant properties although peptides with different disulfide connectivities show a variation of activity. Some of these isoforms display enhanced chemoattractant properties suggesting a role of the topological motifs surrounding the disulfide sites in the receptor binding and activation of HBD-3 (Wu *et al.*, 2003).

The primary structures of HBD1, HBD2 and MIP-3α display very weak homology but a few similarities were identified at the tertiary structural level (Hoover *et al.*, 2002; Perez-Canadillas *et al.*, 2001). The shallow grove between the N-loop and the β2- β3 hairpin potentially create the region and specificity required for binding (Figure 1.7) The N-terminal DCCL motif in MIP-3α plays a role in receptor interactions and it has been postulated that HBD1 and HBD2 carry similar motifs (DHY in HBD-1 and DPV in HBD-2) (Pazgier *et al.*, 2006). Both MIP-3α and the β-defensins share some similarity in the distribution of their positively charged residues on the molecular surface and surprisingly MIP-3α displays antimicrobial properties (Yang *et al.*, 2003).

1.8.3 Additional activities of the defensins

In addition to their direct antimicrobial activity and chemoattractant properties a number of other activities have been observed with the defensins (Figure 1.8). Human,

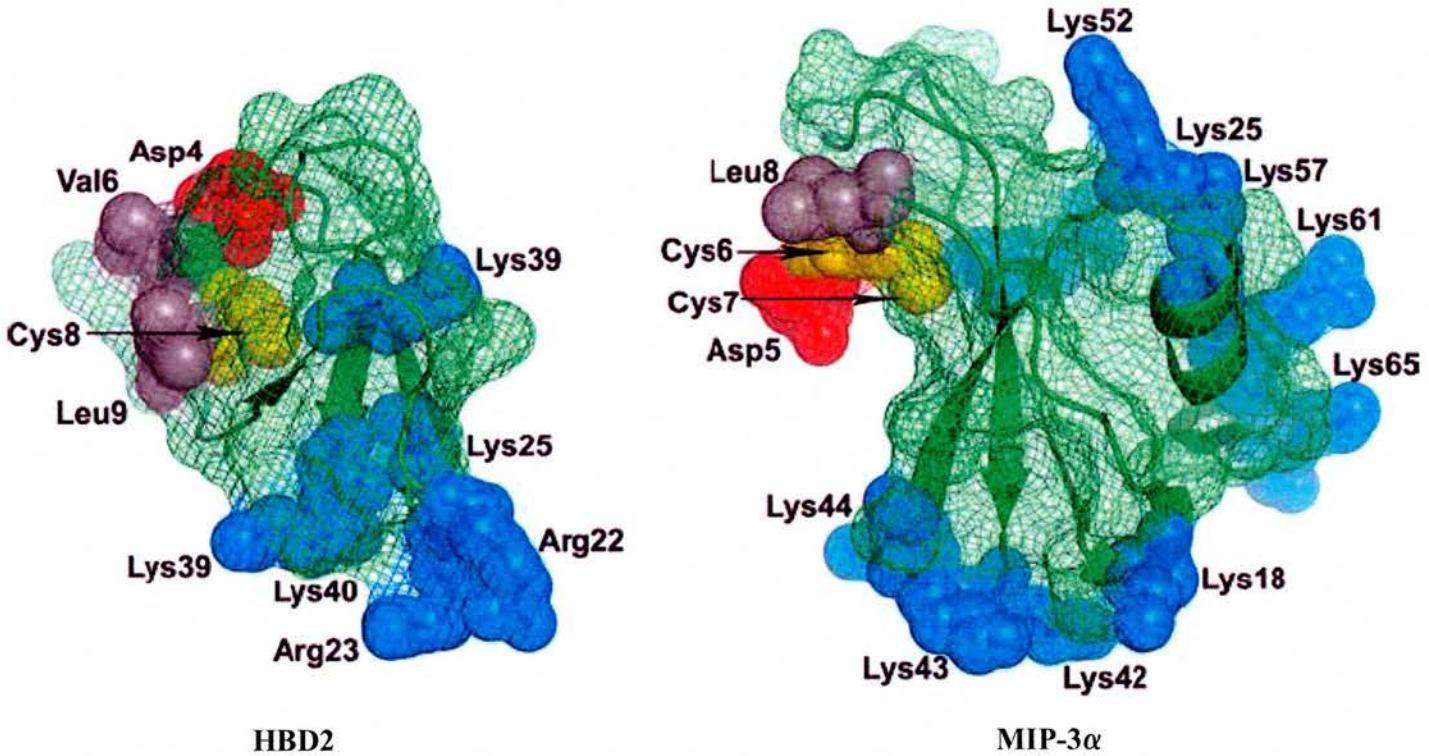


Figure 1.7 Residues reported as important for chemotactic activity against HBD-2 and MIP-3α.

Monomers shown in the form of the backbone tubes sketched together with molecular surfaces. The aspartate residues are highlighted in red, hydrophobic residues (Leu, Tyr or Val) in grey and the positively charged residues in blue. The cysteine residues within the DPVTCL and DCCL motifs of HBD-2 and MIP-3α are shown in yellow.
Figure taken from Pazgier *et al.*, 2006

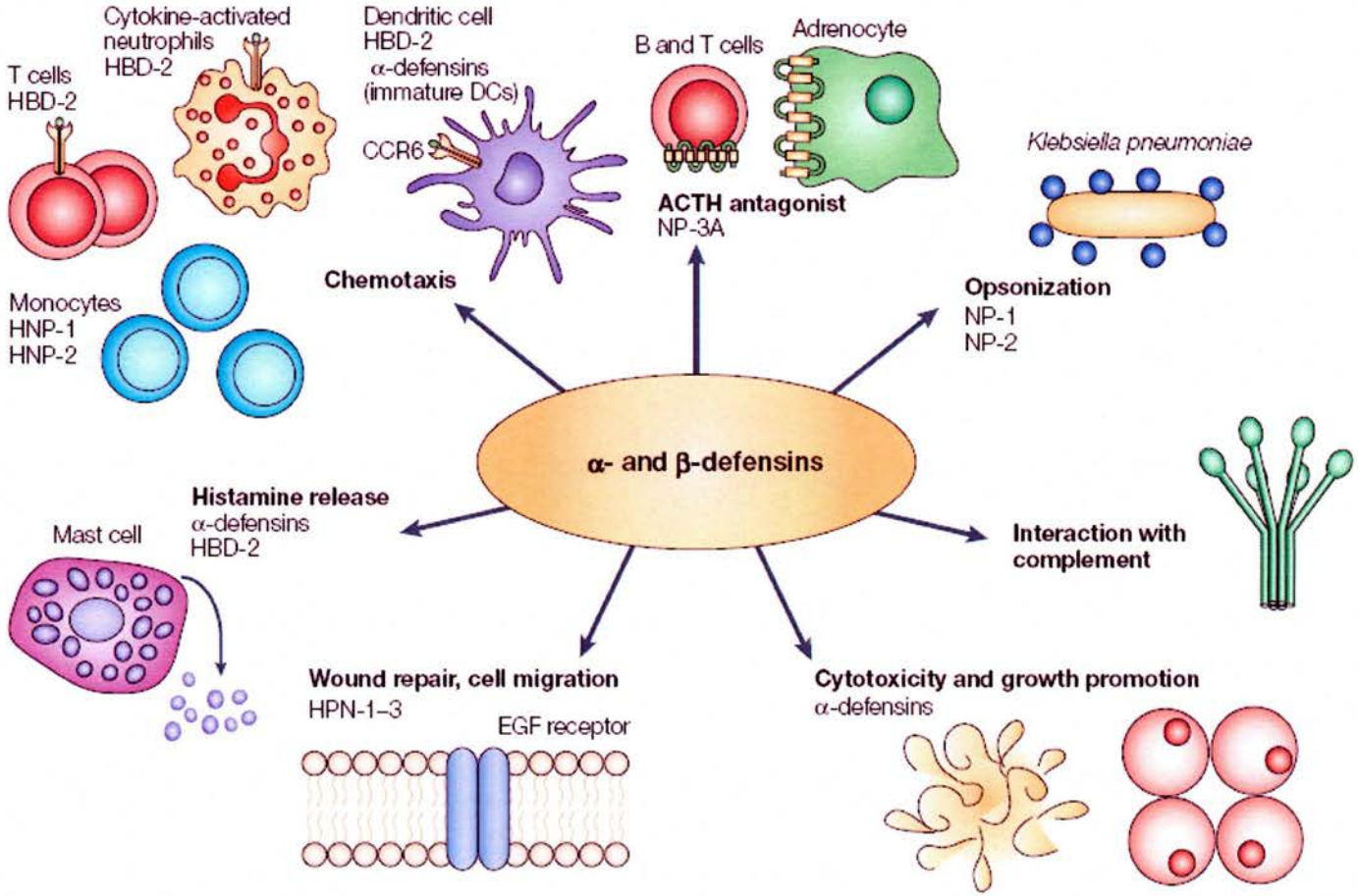


Figure 1.8 Multiple functions of the α- and β-defensins
The defensins display mulitiple functions including chemotaxis, histamine release, wound repair, cytotoxicity and opsonization. (Taken from Lehrer *et al.*, 2004)

rabbit and guinea pig α-defensins are able to induce mast cell degranulation resulting in the release of mast cell granule products including histamine (Befus *et al.*, 1999).

HNP1-HNP3 can augment IL-8 gene transcription and IL-8 production by bronchial epithelial cells (Van Wetering *et al.*, 1997). As mast cell degranulation brings an influx of neutrophils (Malaviya *et al.*, 1996) and IL-8 works as a potent chemoattractant (Baggiolini *et al.*, 1998), antimicrobial peptides may indirectly promote the recruitment and accumulation of neutrophils at inflammatory sites.

Human α-defensins have been shown to display activity with complement. Human α-defensins can bind to complement C1q to either enhance or suppress the activation of the classical pathway in vitro (Prohaszka *et al.*, 1997; van den Berg *et al.*, 1998). Presumable α-defensins exhibit similar activity in vivo in regulating the function of the complement system (Yang *et al.*, 2004).

The α-defensins have also been reported to inhibit the production of immunosuppressive adrenal steroid hormones (Tominaga *et al.*, 1990). α-defensins levels within the plasma can increase during systemic infection and have shown to interfere with the production of adrenal glucocorticoids. As the glucocorticoids are potent immunosuppressive mediators, the α-defensins may also enhance systemic antimicrobial immunity in vivo by inhibiting the production of glucocorticoids (Yang *et al.*, 2004).

The defensins also play an important role within the adaptive imuune system regarding dendritic cells. Immature dendritic cells (iDCs) take up antigens at the site of microbial entry. While processing the antigens the iDCs mature in response to microbial products and mediators to mature dendritic cells (mDCs). The recruitment and maturation of dendritic cells is essential for the induction of the adaptive immune response. Defensins have been found to be direct chemoattractants for iDCs interacting with CCR6 a G-protein coupled chemokine receptor. HBD1 and HBD2 are selectively chemotactic for human iDCs but not mDCs (Yang *et al.*, 1999).

A fusion peptide including mBD2 has been shown to induce the maturation of iDCs into mDCs characterised by the upregulation of CD86, MHC II and CCR7 (Biragyn *et al.*, 2002).

The effect of defensins on DCs and T lymphocytes predicts that they may contribute to the induction of adaptive antimicrobial immunity. The α-defensins have shown in vivo immunoenhancing activity. HNP1-HNP3 can enhance antigen specific immune responses when coadministered with ovalbumin. The production of ovalbumin specific serum IgG antibody and the generation of IFNγ, IL-5, IL-6 and IL-10 providing evidence that the α-defensins can promote humoral and cellular adaptive immune responses (Lillard *et al.*,1999).

The β-defensins have been shown to enhance antigen specific immune responses by the use of a DNA vaccine (Biragyn *et al.*, 2002; Biragyn *et al.*, 2001). mBD2 and 3 were fused with sFv38 a B-cell epitope and used to immunise mice. Potent humoral immune responses against the non immunogenic sFv38 were displayed in addition to antitumor protection.

While advances have been made in the development of defensins as therapeutics, there are still no peptides clinically available. The problems associated with these peptides hinders their current use as potential agents but further investigation will provide knowledge required to develop these agents.

1.9 AIMS OF THESIS

The work in this thesis aimed to analyse the activities of various murine β-defensins in an attempt to relate structure to function.

1. To evaluate the antimicrobial and chemoattractant properties of a novel five cysteine defensin related peptide (Defr1)
2. To analyse Defb14, the murine orthologue of human β –defensin 3 (HBD3)
3. To investigate the properties of Defb14 defensin inspired peptides

CHAPTER 2:

MATERIALS AND METHODS

2.1 Chemicals and oligonucleotides

All chemicals unless otherwise stated were obtained from Sigma Chemical Company (Poole, UK) and were of the highest grade possible.

2.2 Cell lines and handling

See Table 2.1 for list of cell types used. All tissue culture work involving the use of human, animal primary or transformed cells was performed using a vertical laminar flow hood (BionMat^2, Manchester UK). Cells were grown in incubators maintained at 37°C with a 5% CO_2 atmosphere. Confluent cultures of all adherent cells were passaged by trypsin ethylenediamine tetra-acetic acid (trypsin-EDTA). Briefly, cells were washed twice with sterile phosphate buffered saline (PBS) and incubated at 37°C in 2 mL trypsin-EDTA (0.05% trypsin, 0.02% EDTA) for approximately 5 minutes, or until the majority of cells had rounded and detached from the flask. The action of trypsin-EDTA was then blocked with the addition of an equal volume of complete media. Cells were harvested by centrifugation and then resuspended in 10 mL of fresh media before plating.

2.3 Cell Isolation

2.3.1 Neutrophil isolation

The isolation of neutrophils was based on a method from Rot (1991). Adult mice were injected peritoneally with 0.5 mL 9% (w/v) casein solution. A second dose of casein was administered 24 hr later and after a further 3 hours the mice were killed by cervical dislocation. The peritoneal cavity was lavaged using 5mL Dulbeccos modified eagles medium (Invirogen, Paisley UK) DMEM /0.5mM EDTA and the collected fluid placed on ice (*Casein injections, cervical dislocation and lavage performed by Dr Sheila Webb MRC Human Genetic Unit*). To this a further 16 mL of ice cold 0.83% (w/v) ammonium chloride (pH 7.4) were added to the lavage and incubated for 15 min at room temperature. 30 mL of Hanks Buffered Salt Solution HBSS (Invitrogen) were added and the sample centrifuged for 10 min at 880 x *g*. The supernatant was removed and 10 mL HBSS was added, centrifuged for 10 min at 496 x *g* and the cells resuspended in 5 mL HBSS. The sample was

CELL TYPE	DESCRIPTION	SOURCE	MEDIUM USED FOR CULTURE
HEK 293	Human embryonic kidney cell line	American Type Culture Collection (ATCC) VA, USA.	Dulbecco's modified Eagles medium (DMEM)*
HEK 293 CCR6	Human embryonic kidney cell line transfected with chemokine receptor 6	De Yang, Frederick USA.	Dulbecco's modified Eagles medium (DMEM)**

Table 2.1 Cell lines used and media required to culture them

* Supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine and 10 % (v/v) FCS

** Supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 10 % (v/v) FCS and 800 µg/mL geneticin G-418 sulphate

(All culture media purchased from Invitrogen)

centrifuged at 496 x *g* for 10 min. The resulting pellet was resuspended in 500 µl of PBS. A total cell count was performed using a Neubauer hemocytometer chamber (VWR International Ltd, Poole UK) and the percentage of neutrophils estimated from a DiffQuick (Raymond A Lamb Ltd, Eastbourne UK) differentially stained cytospin (Shandon Scientific Ltd, Runcorn UK). Samples with over 90% neutrophils were resuspended in DMEM/0.5mM EDTA for further experiments.

2.3.2 $CD4^+$ T-lymphocyte isolation

$CD4^+$ T cells were isolated by a mini MACS magnetic cell sorter (Miltenyi Biotech, UK) using a positive selection protocol. The mice were killed by cervical dislocation and the spleen removed and placed in PBS. The spleen was homogenised and centrifuged at 496 x *g* for 5 mins. The pellet was resuspended in 1 ml of MACS buffer (PBS, 2mM EDTA, 0.5% BSA & 0.1% sodium azide) and viable cells counts were determined using a haemocytometer and 10% trypan blue solution. To the remaining cells, CD4 (L3T4) magnetic microbeads (Miltenyi Biotec, UK) were added and the cells incubated at 4°C for 15 min. While incubating 500 µl of MACS buffer was added to the LD MACS separation column (Milteny Biotec, UK) on three occasions. After incubation of the cells with the magnetic beads, the suspension was made up to 5 mL using MACS buffer and centrifuged at 496 x *g* for 5 min. After centrifugation, the pellet was resuspended in 250 µl of MACS buffer and added to the magnetic separation column. 2 x 500 µl of MACS buffer were added to the column following the sample to wash through any negative isolation. The column was removed from the magnet and placed over a universal to collect the positive isolation. 2mL of MACS buffer was added to the column and pushed through using the column plunger to collect the sample. The samples were centrifuged and resuspended.

2.3.3 Human monocyte and T cell isolation

Mononuclear cells were isolated from human peripheral blood or bone marrow of normal donors by routine Ficoll-Paque density gradient centrifugation. CD34+ cells were then purified from the resulting mononuclear cells with a magnetic cell sorter CD34 progenitor isolation kit (Miltenyi Biotech) and peripheral blood T cell subsets were purified with human T cell subset enrichment columns (R&D systems). *Protocol performed and optimised by De Yang (Frederick, USA).*

2.4 Chemotaxis Assay

Chemotaxis was perfomed using a 96 well chemotaxis chamber (Neuro Probe Inc, Gaithersburg USA). 30 µl of sample was loaded into the bottom chamber including the positive and negative controls, which were all assayed in triplicate. All murine chemokines (SDF1α, fMLP & CCL20) were purchased from PeproTech EC (London, UK) and dissolved in RPMI / 1% BSA. Negative control for this assay consisted of RPMI / 1% BSA (chemotactic media alone). Stock solutions were prepared at 5 µg/ml. A polycarbonate filter (Neuro Probe Inc, Gaithersburg USA) was added to the top chamber against the silicon gasket and carefully placed on top of the bottom chamber to eliminate air bubbles. The chamber was carefully assembled and incubated at 37 °C while preparation of the cells occurred. Cells were prepared in RPMI / 1% BSA and 225 µl added to the wells in the top chamber at 1-5 x 10^5 cells/mL. The chamber was incubated in a humid 5% CO_2 incubator for a period of time specific to the cell type being studied (Table 2.2). Once incubation was complete the top chamber was removed and inverted. The filter was removed and the side with the non-migrating cells was washed in PBS to remove any cells that may interfere with staining. Wells within the lower chamber were counted to detect any cells that had passed through the filter and failed to adhere to the underside. No cells were detected in the lower wells.

Chemotaxis assays involving human cells were performed by De Yang (Frederick, USA). In addition checkerboard assays, whereby the same concentration of peptide is present in the upper wells in addition to that in the lower wells were performed on all full length peptides except Defb14 all alanine. This was to

CELL TYPE	DESCRIPTION	FILTER PORE SIZE	FILTER CONDITIONS	INCUBATION TIME
HEK 293	Human embryonic kidney cell line	10 μM	Coated overnight with collagen 5 μg/ml 37 °C	4 hrs
HEK 293 CCR6	Human embryonic kidney cells transfected with CCR6	10 μM	Coated overnight with collagen 5 μg/ml 37 °C	4 hrs
$CD4^+$ T cells	Purified from mouse spleens	5 μM	Coated with fibronectin at 1 μg/ml 4 °C for overnight	5 hrs
Neutrophils	Derived from mouse peritoneum	5 μM	Not coated	1.5 hrs
Monocytes *	Purified from human peripheral blood mononuclear cells	5 μM	Coated with fibronectin at 1 μg/ml at 4 °C overnight	1.5 hrs
$CD4^+$ T cells *	Purified from human peripheral blood mononuclear cells	5 μM	Coated with fibronectin at 1 μg/ml 4 °C for overnight	5 hrs

Table 2.2 Cell types and conditions used in chemotactic analysis

*Human cell isolation and human cell chemotaxis performed by De Yang (Frederick, USA).

The underside of the filters were coated with fibronectin or collagen (Sigma) and incubated at 37°C for the required time. Both fibronectin and collagen were dissolved in sterile H_2O.

determine if the migration observed was directed (chemotaxis) or random (chemokinesis). Chemokinesis was not observed only chemotaxis (Personal communication De Yang Frederick, USA).

2.4.1 Staining of filters

For all cell types used the filter was allowed to air dry and subsequently immersed in solution A (methanol fixative) of DiffQuick for 2 min. The filter was removed and stained with solution B (Azure B) for 2 min and solution C (Eosin) for 2 min. Once the staining protocol was complete the filter was gently rinsed in sterile water and allowed to dry.

2.4.2 Filter Counts

Three random fields of view were counted for every well. The mean number of cells migrated and the standard error of the means (SEM) were calculated. In addition counts were performed on the lower wells to assess for cells that passed through the filter and failed to adhere to the underside. No cells were present in the lower wells.

2.5 Haemolysis assays

Erythrocytes were collected in vacuum tubes containing heparin as an anticoagulant. The erythrocytes were harvested by centrifugation for 7 min at 4000rpm and washed three times in Tryptone soya broth (TSB) (Oxoid,Basingstoke UK) supplemented with 287 mM glucose. The pellet was resuspended in TSB + 287 mM glucose and diluted 1:50. 100 µl of the suspension was added to different amounts of the peptide in V-bottomed 96 well plates (Greiner Bio-One Ltd, Stonehouse UK), resulting in peptide concentrations in a range of 3.8 to 500 µg/ml. The plates were incubated for 60 min at 37 °C and centrifuged at 1488 x *g* for 5 min. The supernatants from each well were transferred to a flat bottom 96-well plate (Greiner Bio-One Ltd). The release of haemoglobin from the lysed erythrocytes was measured at 550nm.

The percentage haemolysis was calculated by:

$$\frac{(A_{550} \text{ peptide treated sample} - A_{550} \text{ media treated sample})}{(A_{550} \text{ of Tween-20 treated sample} - A_{550} \text{ media treated sample})} \times 100\%$$

All experiments were performed in triplicate on three independent occasions.

2.6 Tricine gel electrophoresis

2.5 µg of peptide was dissolved in 2.5 µl of 0.01 % acetic acid and 5 µl of 2x sample buffer (Novex Tricine/SDS sample buffer LC1676). Reduction of samples was performed by adding 10 mM of NuPage sample reducing agent (Invitrogen NP0004) and incubated at 80 °C for 5 min. The entire sample was loaded on a 16% tricine gel and run in Novex tricine running buffer (10x) (Invitrogen). The gel was fixed and stained with coomassie blue stain.

2.7 Preparation of RNA

2.7.1 RNA from tissues

All RNA preparations were performed in a double HEPA filter hood (Medical Air Technology, Manchester UK) using RNase and Dnase free filtered tips. Tissue samples were homogenised using an Omni portable homogeniser (Camlab Ltd, Cambridge UK) in 1 mL of RNAzol B (Biogenesis Ltd, Poole UK) until completely lysed. Samples were transferred to a fresh screw top eppendorf to which 100 µl of chloroform was added and vortexed for 15 seconds. The samples were incubated on ice for 5 min and centrifuged at 20380 x *g* for 15 min at 4 °C and the top layer carefully transferred to a fresh tube on ice without disturbing the lower layer. To this an equal volume of ice cold isopropanol was added and incubated on ice for 15 min. The samples were centrifuged at 20380 x *g* for 15 min and the RNA pellet was washed with 75 % (v/v) ice-cold ethanol and allowed to dry. The RNA was resuspended in 50µl DEPC treated water and assessed by spectrophotometry. The ratio between the absorbance values A_{260}/A_{280} gives an estimate of the RNA

purity. A ratio of 2.0 is observed for pure RNA. Samples with lower ratios were discarded. Ratios, concentrations and absorbance were all calculated automatically by the spectrophotometer.

2.7.2 DNase treatment of RNA

To remove any DNA present in the RNA samples, Dnase 1 RNase-free (Roche) was used. The samples were treated using 10 x Dnase 1 buffer, 10 units Dnase 1 and 40 units of RNA inhibitor. The samples were incubated at 37 °C for 60 min and then extracted using an equal volume of phenol:chloroform, vortexed and centrifuged at 20380 x *g*. The top layer was carefully transferred to an equal volume of chloroform, vortexed and centrifuged at 20380 x *g* for 15 min. The top layer was transferred to a fresh tube on ice and 0.1 volumes of 3 M sodium acetate (pH 5.6) and 2 volumes of ethanol were added. The samples were incubated at -70 °C for 30 min. The sample was centrifuged at 20380 x *g* for 15 min and the pellet washed in 70 % (v/v) ethanol. The pellet was dried and resuspended in DEPC H_2O.

2.8 Amplification of RNA and DNA by Polymerase Chain Reaction

All PCR reactions were performed using filtered RNase and DNase free tips.

2.8.1 Amplification from RNA

Amplification of RNA was carried out using a first strand cDNA synthesis kit for RT-PCR (AMV) Roche UK. Duplicate samples of 2 µg of RNA in a total volume of 7.8 µl DEPC treated H_2O were denatured for 15 min at 65 °C. To one set of samples

2 µl of 10 x PCR buffer
4ul $MgCl_2$
2 µl 10 mM dNTPs
0.4 µl gelatin
2 µl of random primer p(dN)6
40 units RNase inhibitor

0.8 µl DEPC treated H_2O or 0.8 µl AMV reverse transcriptase

RNA minus sample was included in the set as a control for contamination. The samples were incubated at RT for 10 min to allow the primers to anneal then incubated at 42 °C for 60 min followed by 5 min at 95 °C to inactivate the AMV. Once cooled 5 µl was removed and used in the PCR reaction described next.

2.8.2 Amplification from DNA

1 µg cDNA

5 µl 25 mM $MgCl_2$

5 µl 10 x PCR buffer

5 µl DMSO

1 µl of 50 x dNTP mix (10 mM of each nucleotide)

1 µl of 100 mM forward and reverse primers

0.5 µl of thermostable DNA Taq polymerase

(All PCR reagents were purchased from Invitrogen)

The above were added to a thin wall eppendorf. A programmable heating block (OmniGene, Hybaid) controlled thermal cycling. The following PCR reaction was utilised for all the defensins genes with the exception of varying annealing temperatures that differed depending on the melting point of the primers (Table 2.3).

PCR cycle conditions

Step 1: 94 °C for 3 min

35 cycles of:

Step 2: 94 °C for 1 min

Step 3: (annealing temperature) for 30 seconds

Step 4: 72 °C for 1 min

Final Step 5: 72 °C for 10 min

RT-PCRs performed by Dr Alison Maxwell MRC Human Genetics Unit

PRIMER	SEQUENCE	TARGET	ANNEALING TEMP (°C)	PRODUCT SIZE (bp)
Defb14 F	5' TCT TGT TCT TGG TGC CTG CT 3'	*defb14*	54	166
Defb14 R	5' TTC TTC TTT CGG CAG CAT TT 3'	*defb14*	54	166
HPRT F	5' CTG TAG ATT TTA TCA GAC TGA AGA G 3'	*hprt*	54	299
HPRT R	5' GTC AAG GGC ATA TCC AAC AAC AAA 3'	*hprt*	54	299
Defb14 *	5' GGA CGC ATT CCT ACC AAA AA 3'			

Table 2.3 Oligonucleotide sequences

* Used as internal radiolabelled oligonucleotides

(All oligonucleotides purchased from Invitrogen)

2.83 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using gel apparatus (Bioscience Services). Gels were prepared at 1 – 1.5 % agarose depending on the resolution required. The agarose was dissolved in TBE buffer (0.09 M Tris-borate, 0.002 M EDTA (pH 8.0) and ethidium bromide (5 μg/100 ml)(BioRad). Gels requiring resolution of DNA fragments below 200bp used Nu-SIEVE agarose gel (FMC Bio Products). Gels were run in TBE buffer at 100 V for 90 min and bands detected using a UV illuminator.

2.84 Southern blot transfer of DNA

Once the DNA fragments were separated by gel electrophoresis the gel was soaked in denaturing buffer (0.5 M sodium hydroxide and 1.5 M sodium chloride) for 20 min. The gel was transferred and soaked in neutralising buffer (0.5 M Tris, 1.5M sodium chloride) for 10 min. The gel was then transferred onto Hybond-N (Amersham Biosciences) by capillary transfer overnight in 20 x SSC (3M sodium chloride, 0.3M sodium citrate). The membrane was removed and blotted dry before being UV stratalinked (Stratalinker 1800, Stratagene).

2.9 Radioactive Hybridisation

2.9.1 Preparation of radioactively labelled oligonucleotide probes

50 ng of oligonucleotide probes (15-25bp in length) were labelled in a total volume of 20 μl. 2 μl of 10 x polynucleotide kinase (PNK) buffer, 10 units of PNK and 30 μCi [γ-32P] dATP. The reaction was incubated for 1 hour at 37 °C and then added to the prehybridised filter.

2.9.2 Hybridisation of radioactive oligonucleotide probes

Filters were pre-hybridised at 48 °C for 4 hours in a rotating hybridisation bottle. This contained 20-30 mL oligonucleotide hybridisation buffer (3 M sodium chloride, 0.3 M trisodium citrate, 0.4 % SDS and 0.2 % sodium pyrophosphate) and 1 mg of sonicated salmon sperm. After pre hybridisation, radiolabelled

oligonucleotides were added directly to the bottle and the solution and blots hybridised for 4 hour at a temperature 4-6 °C lower than the melting temperature of the oligonucleotide. Filters were then washed with 6 M sodium chloride, 0.6 M trisodium citrate and 0.1 % SDS. The filters were placed in a cassette and exposed to Kodak X-Omat AR film at –70 °C.

2.10 Bacterial killing assays

Test organisms (Table 2.4) were grown to mid-logarithmic phase in Iso-Sensitest broth (ISB) (Oxoid) growth media and then diluted to 1-5 x 10^6 colony-forming units (CFU)/ml in 10 mM potassium phosphate containing 1% (v/v) Iso-Sensitest broth, pH 7.4. Different concentrations of test peptide were incubated in 100 µl of cells (1–5 x 10^5 CFU) at 37 °C for 3 h. 10-Fold serial dilutions of the incubation mixture were plated on Iso-Sensitest agar (ISA) plates, incubated at 37 °C, and the CFU determined the following day. The minimum bactericidal concentration (MBC) is the concentration of peptide where we observed >99.99% killing of the initial inoculum. All assays were performed in triplicate on three independent occasions. The MBC was obtained by taking the mean of all the results, and experimental errors were within one doubling dilution. Assays involving DTT require 10 mM of Nupage sample reducing buffer (Invitrogen) to be added to the peptide stocks before commencing the bacterial killing assays.

2.10.1 Salt sensitivity killing assays

To the 10 mM potassium phosphate buffer + 1 % ISB, sodium chloride was added between 0 mM - 250 mM and applied to each individual peptide concentration. The assay was performed as described above.

2.10.2 Serum killing assays

To the 10 mM potassium phosphate buffer + 1 % ISB, human serum (pooled, heat inactivated) was added (ICN Biomedicals, USA). Various percentages 0.1, 1 & 10 % were evaluated at each individual peptide concentration. The assay was performed as described in the bacterial killing section.

ORGANISMS	STRAINS	DESCRIPTION	SOURCE
Acinetobacter baumannii	ATCC 19606 U45	Gram negative rods	CF laboratory
Bordetella bronchiseptica	J3083	Gram negative coccobacillus	CF laboratory
Burkholderia cenocepacia	J2315 ATCC 25416	Gram negative rods	CF laboratory
Candida albicans	J2922	Fungi	CF laboratory
Escherichia coli	ATCC 25922	Gram negative rods	CF laboratory
Enterococcus faecalis	ATCC 700802	Gram positive cocci	CF laboratory
Pseudomonas aeruginosa	PAO1,C3425, H183, C4269, C3781	Gram negative rods	CF laboratory
Ralstonia eutropha	C3081	Gram negative rods	CF laboratory
Ralstonia pickettii	C3079	Gram negative rods	CF laboratory
Staphylococcus aureus	ATCC 25923	Gram positive cocci	CF laboratory
Staphylococcus aureus MRSA	J2918	Gram positive cocci	CF laboratory
Stenotrophomonas maltophilia	C1980, C3625, C3626, C3627	Gram negative rods	CF laboratory

Table 2.4 Microorganisms used in bacterial killing assays

Cystic Fibrosis Microbiology Laboratory and Strain Repository, Medical Microbiology Division, Edinburgh University Medical School, Edinburgh, UK

(All bacterial agar and culture media purchased from Oxoid)

2.11 Peptide Synthesis

All peptides were chemically synthesised by standard solid phase methodology. Defb14, Defb14 1 cys, hBD3, Defr1, Defr1 1 cys & 5YC were obtained from Chemical Synthesis Services-Albachem Ltd (Gladsmuir, UK). The Defb14 inspired peptides were made in-house using automated peptide synthesis (Applied Biosystems 433A peptide synthesizer) using Rink amide AM resin for peptide amides, pre-loaded NovaSyn TGT resin for peptide acids and Fmoc amino acids (Novabiochem). The identity and purity of all peptides was confirmed by LC-mass spectra (Micromass Quattro LC mass spectrometer). Semi-preparative HPLC was performed using a Phenomenex Luna C18 column and a gradient of 5-95% acetonitrile (containing 0.1% TFA) over 45 min (flow rate of 3.0 mL/min). Automated solid phase synthesis was carried out on a 0.05 mmol scale using 0.5 mmol of each Fmoc amino acid per coupling reaction and HBTU/HOBt as coupling reagents. The coupling time was 0.5 h. Peptide products were cleaved from the resin with 95 % TFA, 2.5 % ethanedithiol, 2.5 % water for 3 hours. The resin was filtered-off, washed with TFA, and filtrate poured into diethylether (10 volumes), centrifuged for 3000 rpm for 15 min, resuspended in ether and re-centrifuge for 3000 rpm for 15 min. The crude peptides were dissolved in water and loaded directly onto a semi-preparative HPLC column. Peptide fractions were identified by mass spectrometry and lyophilised. *Protocol optimised and performed by Derek MacMillian, Department of Chemistry, University College London, London.*

2.12 Mass spectrometry measurements

Accurate mass measurements were performed using a 9.4 T FT-ICR mass spectrometer (Bruker Daltronics). The peptides were electrosprayed at a concentration of 20 μM from a solution of 50:49:1 water/methanol/acetic acid (v/v). The resulting spectra were calibrated externally using 20 μM ubiquitin. A Q-TOF mass spectrometer (Micromass) was employed to obtain masses of all peptides. All peptides were first prepared in 10 mM ammonium acetate, pH 6.4 and analysed by nano-electrospray MS. *Protocol optimised and performed by Bryan McCullough, School of Chemistry, University of Edinburgh, Edinburgh.*

2.13 Disulfide connectivities

The disulfide connectivities were determined by proteolysis and peptide mass mapping. A 50 μM solution of the peptide was subjected to proteolytic cleavage with trypsin (for Defb14) and a combination of trypsin and chymotrypsin for HBD3. The digested peptides were analysed using LC-MS (Micromass Platform I) and by nano electrospray using a Q-ToF (Micromass). For analysing HBD3, a MALDI-TOF mass spectrometer (Voyager Discovery (Applied Biosystems)), and a FT-ICR MS were used. *Protocol optimised and performed by Dave Clarke, School of Chemistry, University of Edinburgh, Edinburgh.*

2.14 Nuclear Magnetic Resonance

The 1D 1H NMR spectra of the peptides were measured using a Bruker 600 MHZ Avance NMR spectrometer equipped with a 5mm triple resonance cryoprobe with z-gradients. Samples (250 μg) were dissolved in 550 μl of a 9:1 mixture of H2O:D2O. The pH was adjusted to 3.5 and 512 and 1024 scans were accumulated at 298 °C in each spectrum. A double pulse field gradient spin-echo was used to suppress the water. *Protocol optimised and performed by Dusan Uhrin, School of Chemistry, University of Edinburgh, Edinburgh.*

2.15 Defr1 HPLC fractions

The peptide was fractioned on a gradient of 20-30%B in 60mins on a Jupiter C5 250 x 10mm semi prep column at a flow rate of 5l/min. 5ml fractions were collected throughout the region of interest and lyophilised and redissolved in 1 ml of water. *Protocol optimised and performed by Albachem Ltd, Elvingston Science Centre, Gladmuir, East Lothian.*

2.16 Statistical analysis

Regarding chemotactic analysis the counts from each well in the chamber were classified by agent, experiment and concentration. The data were transformed to log (x) and ((x+3/8)). Means were taken from both the transformed and untransformed data classified by experiment, agent and concentration to which were

tested for homogeneity of variance between each of the treatment groups. A preliminary analysis of variance was done including all main effects and two-factor interactions of the factors experiment, agent and concentration. When either of the two-factor interactions with experiment were not significant ($p>0.2$) they were removed from the model to help to increase the degrees of freedom available for estimating error. Analysis of variance was then repeated for each case and produced a table classified by the factors agent x concentration, which displayed means, standard errors of means and Minimum Significant (at 5%) Difference (MSD) all shown graphically.

Regarding antimicrobial analysis the data were classified by factors of serum concentration both treated and untreated with the peptide and the number of replicate experiments. Between group variances were stabilised with $\log_e(1+x)$ transformation). The data were transformed and averaged over the replicate experiments. A three-factor balanced analysis of variance was done using the three-factor interaction as the error term. When other two-factor terms were found to be non significant they were removed from the model and fitted valued recalculated increasing the number of degrees of freedom for error and reducing the confidence range. Tables of the transformed data displaying standard errors were produced and represented graphically. All statistical analyses were performed using either Minitab for Windows (Minitab Inc, Philadelphia, USA) or Microsoft Excel (Microsoft Corporation, USA). Data was considered significant in all cases at $p<0.05$. *Statistical advice and analysis performed by Peter Teague, MRC Human Genetic Unit, Edinburgh.*

CHAPTER 3:

ANALYSES OF A NOVEL FIVE CYSTEINE DEFENSIN RELATED PEPTIDE (Defr1)

3.1 INTRODUCTION

Mammalian host defence peptides are diverse in size, structure and function. Over a thousand peptides have been identified from various sources and this continues to expand. Differences in net charge, hydrophobicity and sequence of the peptides allow efficient characterisation and subgrouping of families.

Various peptides have been shown to contain cysteine residues which in turn are responsible for numerous disulfide bonds. With the β-defensins the cysteine residues are reported to be essential for chemoattractant properties but not necessarily for the antimicrobial components (Wu *et al.*, 2003). Multiple families display disulfide bonds within their peptides varying in the number and the position. The mammalian antimicrobial peptide lactoferricin is a 25 residue antimicrobial peptide derived from the N-terminus of lactoferrin (Bellamy *et al.*, 1992) by gastric proteolytic cleavage. This peptide contains one disulfide bond and displays potent antimicrobial activity through its homologues with bovine lactoferricin being the most potent (Vorland *et al.*, 1998). Protegrin derived from porcine leukocytes contain two disulfide bonds reported to stabilize the β-sheet structure. This peptide is an 18 residue broad spectrum antimicrobial agent with activity against the highly resistant *Neisseria gonorrhoeae* (Qu *et al.*, 1997). Hepcidin, a urine antimicrobial peptide predominantly expressed in the liver (Park, et al 2001) contains four disulfide bonds. Hepcidin has been shown to display both antifungal and antibacterial properties.

The β-defensins exhibit three disulfide bonds within their peptide structure. The majority of β-defensins display these properties although exceptions can be found. An intragenic polymorphism of the *DEFB1* gene changes a highly conserved cysteine residue to a serine residue (Circo *et al.*, 2002) As a result of this coding one cysteine remains unpaired and HBD1 is unable to form three disulfide bonds. A synthetic HBD-1 with the variant serine in place of the cysteine residue has been synthesised and tested for antimicrobial properties. The variant HBD-1 still retains antimicrobial properties against *P.aeruginosa* and *C.neoformans* comparable with that of the parent HBD-1.

A murine β-defensin gene present in C57Bl6 mice has been discovered to encode a peptide with only five cysteine residues (Morrison *et al.*, 2002). This peptide Defr1

displays a tyrosine in place of the first cysteine and yet retains potent antimicrobial activity against gram positive and negative organisms. This gene is a variant allele of *Defb8* which encodes six cysteines and is present in all the other inbred murine strains tested. *Defb8* has very high levels of identity (98%) with *Defr1* differing only in three amino acids residues (Bauer *et al.*, 2001).

The expression of Defr1 was analysed extensively in various murine tissues and detected prominently in the heart, testis and to a lesser extent the uterus. Previously Defr1 was synthesised and tested for activity. Morrison *et al.*, 2002 reported that a synthetic Defr1 peptide displayed antimicrobial activity with *S.aureus, E.coli* and *B.cenocepacia* and this was subsequently diminished in the presence of various salt concentrations with the exception of *P.aeruginosa* to which Defr1 retained its antimicrobial properties. Recently the relationship between the structure and activity of Defr1 and its six cysteine analogue has been studied (Campopiano *et al.*, 2004). The peptide 5YC involves reinstating the cysteine residue at the proposed site which is currently occupied with a tyrosine residue. By using electrophoresis and mass spectrometry Defr1 and 5YC were shown to be defensin dimers, Defr1 mediated by an intermolecular bond and 5YC three intramolecular bonds (Campopiano *et al.*, 2004). Connectivities of both were conducted using mass mapping and concluded that 5YC showed the β-defensin disulfide bond connectivity whereas the oxidised Defr1 is a mixture of different dimeric isoforms with unknown inter- and intramolecular connectivities. The differences in antimicrobial properties were studied against *P.aeruginosa*. Oxidised Defr1 displayed significantly more activity than its reduced form and that of both the oxidised and reduced 5YC. Further analysis involved varying salt concentrations in the antimicrobial assays. Defr1 displayed antimicrobial activity at 150 mM NaCl whereas 5YC was inactive at that concentration.

3.2 AIMS

The aims of this chapter were to:

1 Evaluate the antimicrobial properties of Defr1 and 5YC against a panel of microorganisms, including multi resistant isolates

2 To analyse Defr1 fractions collected by HPLC for antimicrobial properties

3 To examine the chemoattractant properties of Defr1 and 5YC

4 To evaluate the synthetic single species Defr1 1cys for effects on functionality

3.3 RESULTS

A synthetic preparation of Defr1 was examined in order to characterise the antimicrobial (Table 3.1) and chemoattractant properties (Table 3.6). Defr1 HPLC fractions were collected to examine for antimicrobial activity. A six cysteine analogue 5YC which involved reinstating the cysteine residue at the proposed site which is currently occupied by a tyrosine residue was studied for antimicrobial and chemoattractant properties. In addition a one cysteine variant Defr1 1cys was created to validate the essentiality of a covalent dimer structure in functionality.

3.3.1 Antimicrobial activity of Defr1 and 5YC against a panel of multi-resistant strains

Antimicrobial analysis was performed on both Defr1 and 5YC (Table 3.1) against a panel of micro-organisms, Gram positive, negative bacteria and fungi (Figure 3.1-3.5). Defr1 displayed potent antimicrobial activity against a spectrum of organisms (Table 3.2). The majority of organisms gave MBC values with Defr1, 3-10 μg/ml with the exception of >100 μg/ml for *B.cenocepacia*. Defr1 gave notable activity against the multi resistant pathogens, *A.baumannii* 3 μg/ml ($p<0.01$), *S.maltophilia* 12.5 μg/ml and *P.aeruginosa* PAO1 6 μg/ml ($p<0.01$). 5YC displayed poorer activity against the different isolates (*A.baumannii* 12.5 μg/ml ($p<0.01$), *P.aeruginosa* 50 μg/ml ($p<0.01$) and *C.albicans* 25 μg/ml ($p<0.01$)) requiring higher concentrations of the peptide to give complete killing.

3.3.2 Gel electrophoresis of Defr1 fractions

In order to characterise Defr1 HPLC fractions were collected (Procedure performed and optimised by Albachem Ltd, (Appendix 1)). As Defr1 is a complex mix the aim of the fractions was to isolate specific isoforms from the mix in hope of displaying various activity profiles. Fractions were analysed by gel electrophoresis on a 16% tricine gel alongside a sample of Defr1 from the previous preparation (Figure 3.6). Defr1 revealed a prominent upper band at around 7kDa and a faint lower band migrating around 4kDa. The fractions run gave clear differences throughout the two gels. The

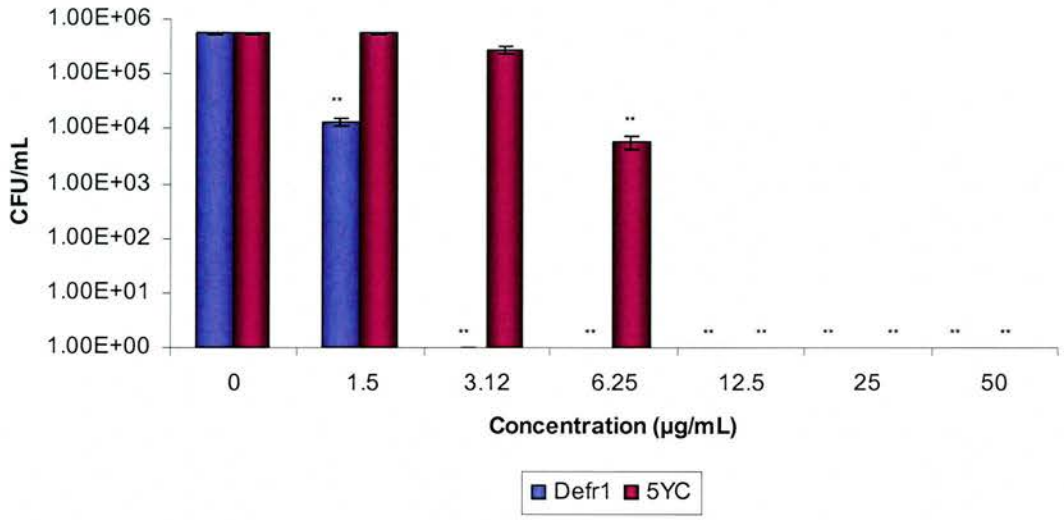
PEPTIDE	SEQUENCE	+
Defr1	DPVTYIRNGGICQYRCIGLRHKIGTCGSPFKCCK	+ 6 (+12)
5YC	DPVTCIRNGGICQYRCIGLRHKIGTCGSPFKCCK	+ 6 (+12)
Defr1 1cys	DPVTYIRNGGIAQYRAIGLRHKIGTAGSPFKCAK	+ 6 (+12)

Table 3.1 Defr1, 5YC and Defr1 1cys peptide sequences

Comparison of synthetic Defr1, 5YC and Defr1 1cys peptide sequences displaying changes in the number of cysteine residues. Amino acids highlighted in red dentote the changes from a cysteine residue to a tyrosine residue. Those highlighted in blue display the conserved cysteine residue. The amino acids highlighted in purple denote the changes from cysteine to alanine in the Defr1 1cys.

+ indicated net charge of monomer and number in brackets indicates the charge of the dimer

A



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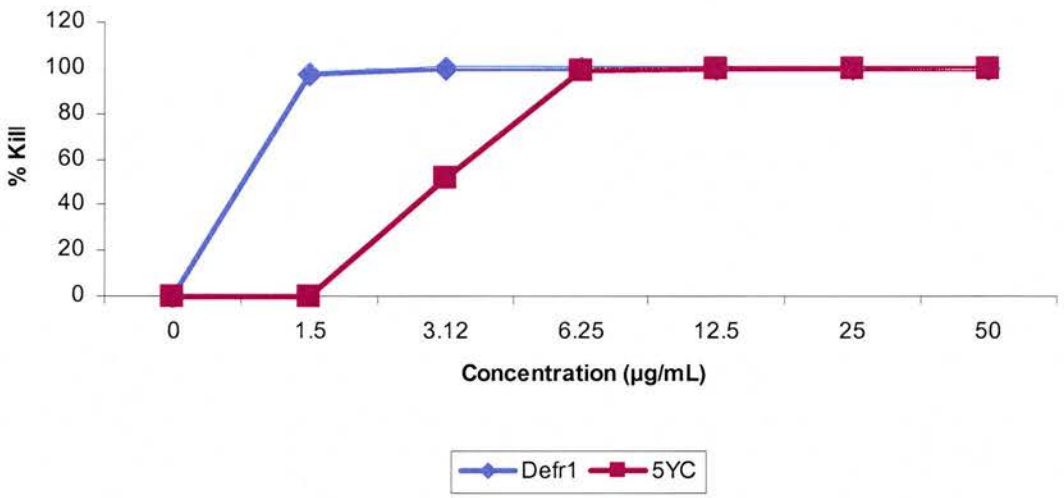
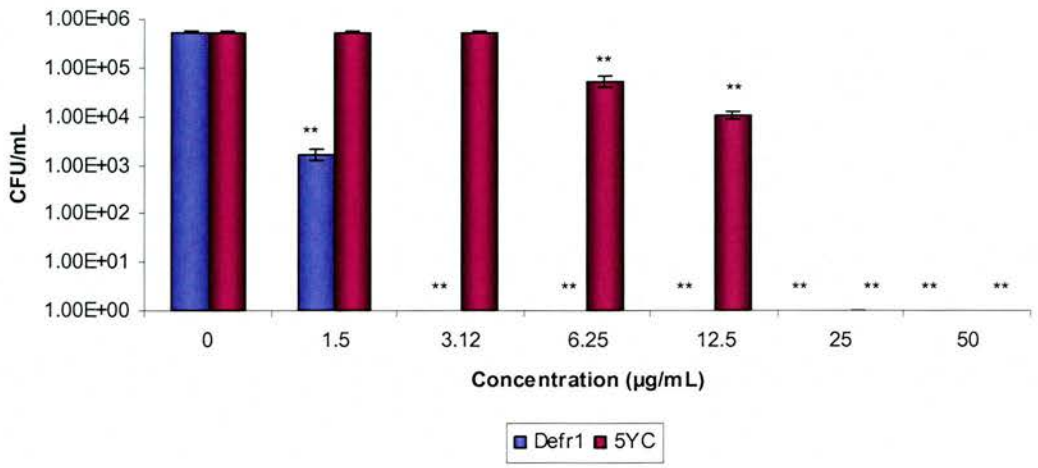


Figure 3.1 Antimicrobial activity of Defr1 and 5YC against *A.baumannii* U45

Graph A shows the mean number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

A



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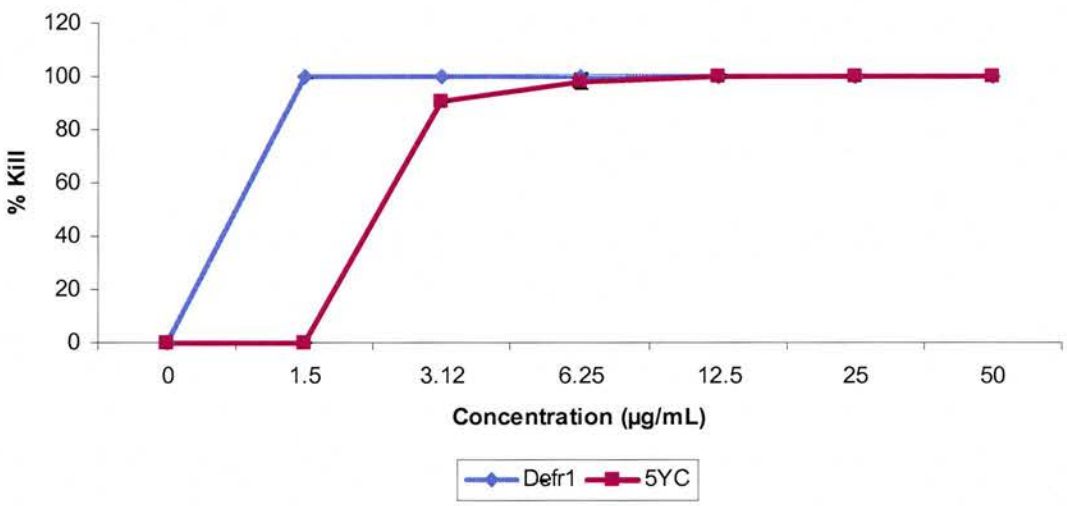
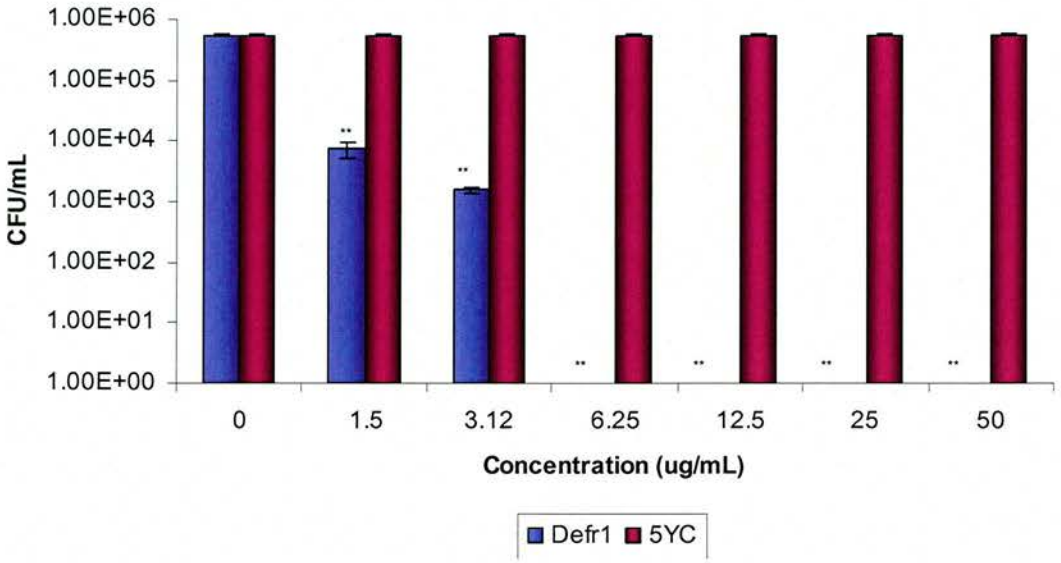


Figure 3.2 Antimicrobial activity of Defr1 and 5YC against *C.albicans* J2922

Graph A shows the mean number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

A



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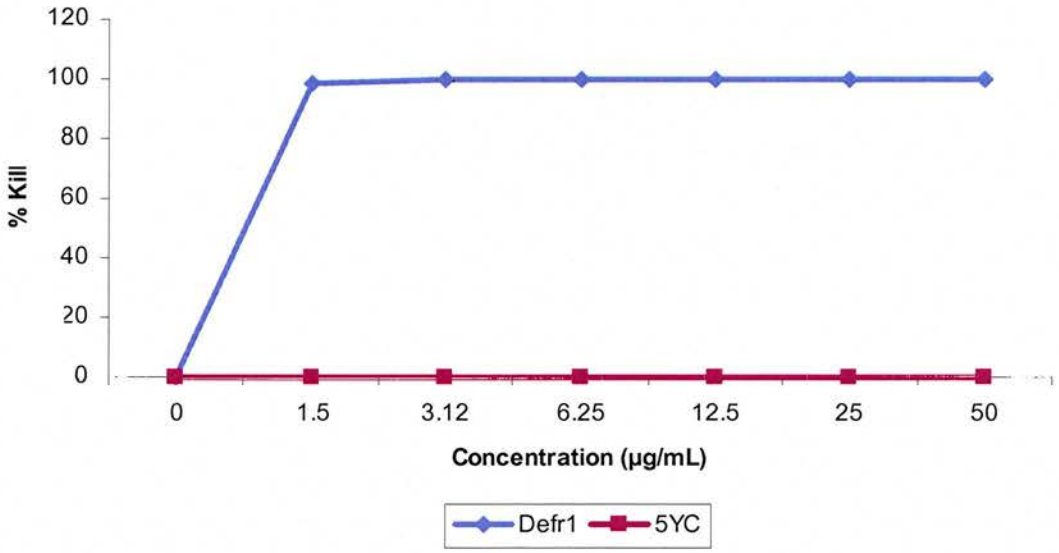
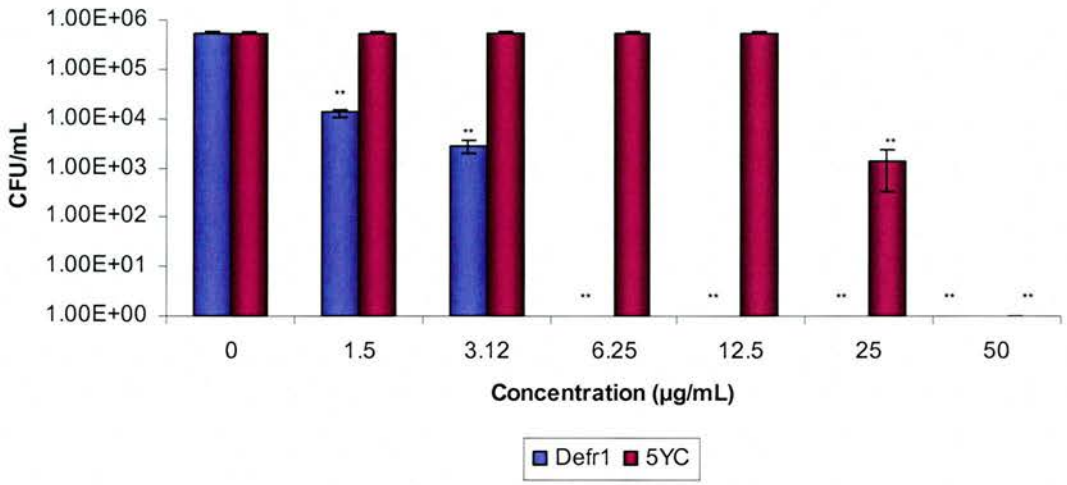


Figure 3.3 Antimicrobial activity of Defr1 and 5YC against *E.faecalis* ATCC 29212
Graph A shows the mean number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

A



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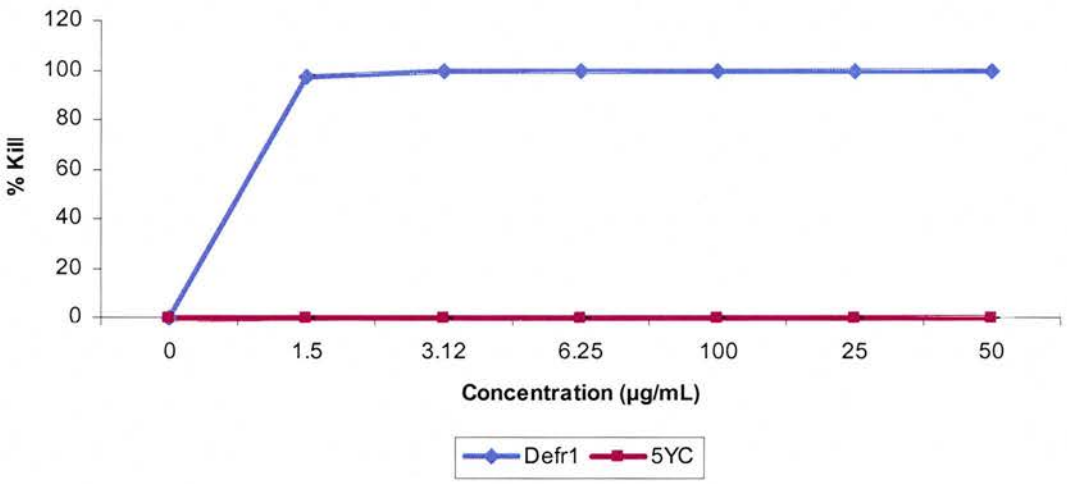
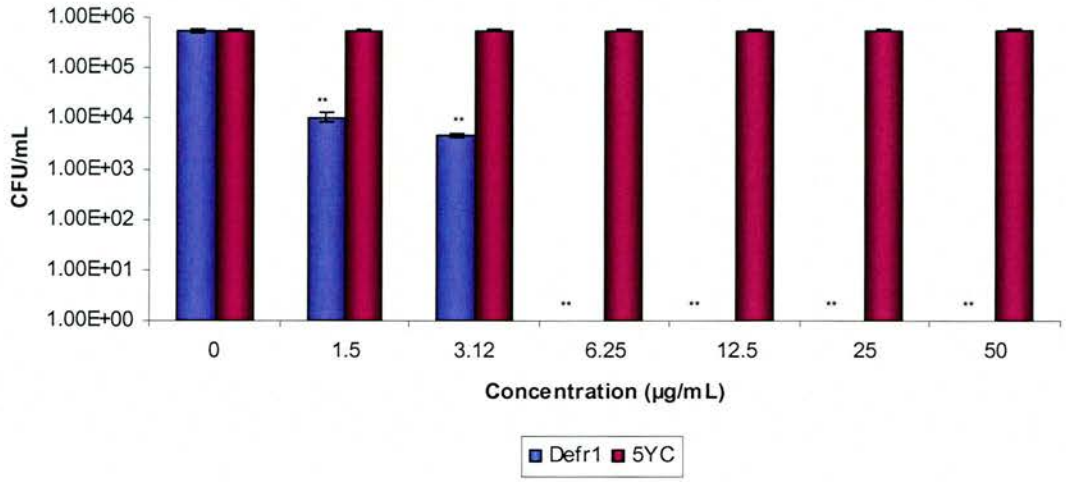


Figure 3.4 Antimicrobial activity of Defr1 and 5YC against *P.aeruginosa* PAO1

Graph A shows the mean number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

A



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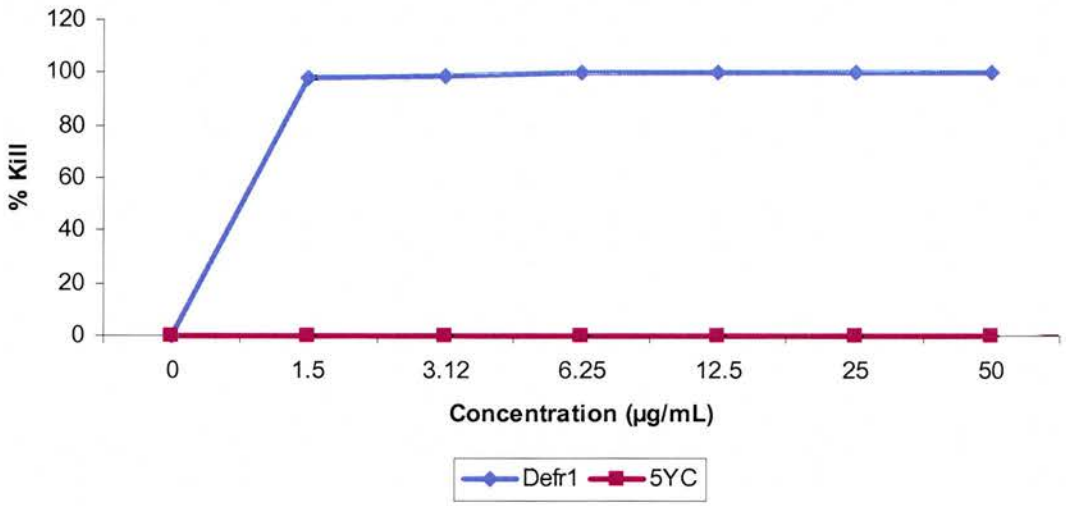


Figure 3.5 Antimicrobial activity of Defr1 and 5YC against *S.aureus* MRSA J2918

Graph A shows the mean number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

ORGANISM	STRAIN	DESCRIPTION	MBC VALUE (µg/ml)		
			Polymyxin	Defr1	5YC
Gram-negative					
P.aeruginosa	PAO1	Type strain	0.5	6	50
	C3425	Manchester	1	6	50
	H183	Liverpool	1	6	100
	C4269	Brisbane	1	6	>100
	C3781	Melbourne	0.5	6	100
E.coli	ATCC 25922	Type strain	0.25	8	>100
B.cepacia	ATCC 25416	Type strain	>512	>100	>100
B.cenocepacia	J2315/LMG16656	Type strain	>512	>100	>100
S.maltophilia	C1980	Clinical isolate	32	12.5	>100
	C3625	Clinical isolate	32	6	>100
	C3626	Clinical isolate	32	3	>100
	C3627	Clinical isolate	64	6	>100
B.bronchoseptica	J3083	Clinical isolate	0.12	6	>100
R.pickettii	C3079	Clinical isolate	>512	3	>100
R.eutropha	C3081/LMG1199	Clinical isolate	128	3	>100
A.baumannii	U45	Clinical isolate	1	3	12.5
Gram-positive					
E.faecalis	ATCC 29212	Type strain	256	6	>50
S.aureus	ATCC 25923	Type strain	256	10	>100
	S113/J2918	Clinical isolate - MRSA	64	6	>50
Fungi					
C.albicans	J2922	Clinical isolate	>512	3	25

Table 3.2 Antimicrobial activity of Defr1 and 5YC against a spectrum of organisms

The antimicrobial activity of Def1, 5YC and polymyxin (positive control) were evaluated against a panel of microorganisms. Minimum bactericidal concentrations were performed by Dave Clarke, Ross Langley and Karen Taylor at the Cystic Fibrosis Microbiology Laboratory, Edinburgh.

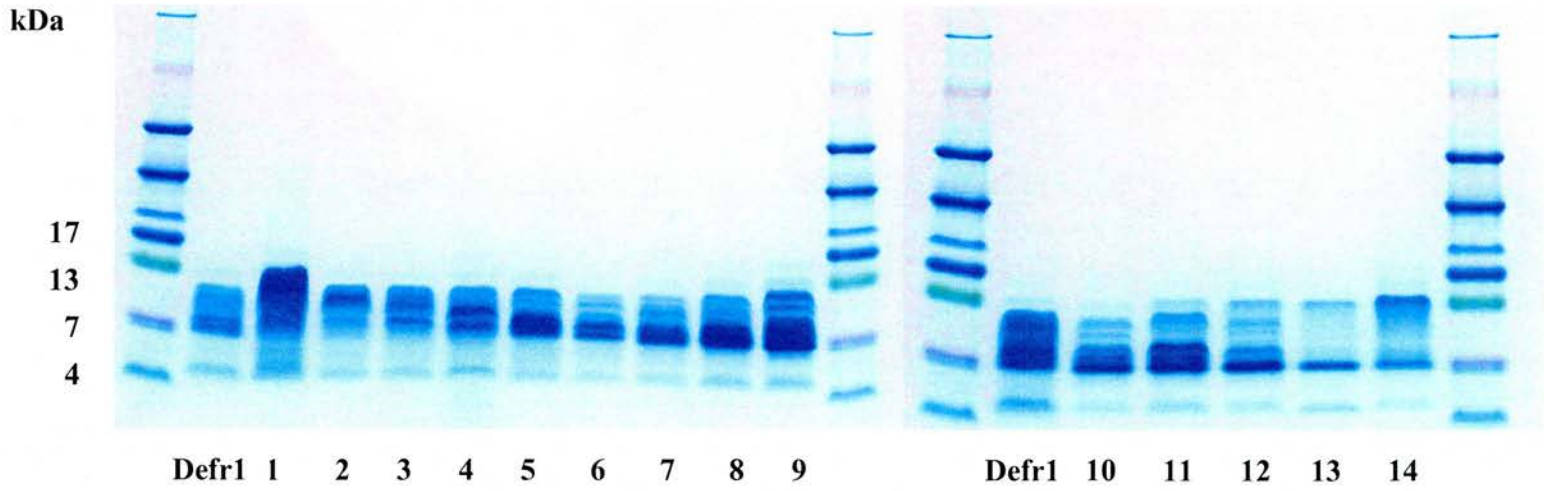


Figure 3. 6 Gel Electrophoresis of Defr1 fractions

Defr1 HPLC fractions 1-14 were collected (Appendix 1) and run on a 16% tricine gel in an attempt to isolate specific isoforms. In addition a preparation of the original Defr1 peptide was run as a comparison. Defr1 revealed a prominent upper band around 7kDa and a faint lower band migrating around 4kDa. The HPLC fractions (1-14) give clear differences throughout the two gels with changes in intensity of the upper 7kDa band displayed.

7kDa upper band change in intensity throughout the samples and towards the end, fractions 10-14, the band diminished in thickness.

3.3.3 Antimicrobial activity of Defr1 fractions

Due to the clear differences observed in the above gels, the antimicrobial activity was analysed to ascertain any differences between the fractions. All fractions were tested against *P.aeruginosa* (Table 3.3) giving MBC values of 6 μg/ml. No differences were detected between the fractions.

3.3.4 Mass spectrometry of Defr1 fractions 2, 9 and 13

Due to the differences visable by gel electrophoresis, 3 fractions were chosen for mass spectrometry. Fractions 2, 9 and 13 were analysed by Q-ToF and performed by Bryan McCullough (Figure 3.7). All three fractions revealed similarities, all showing dimeric properties similar to the original Defr1 preparation (personal communication Perdita Barran).

3.3.5 Antimicrobial activity of Defr1 fractions 2, 9 and 13

Fractions 2, 9 and 13 were reduced with DTT to determine if a change from oxidised to reduced form would alter the antimicrobial properties against *P.aeruginosa* PAO1 (Table 3.4). Antimicrobial activity was abolished with the presence of DTT (Figure 3.8). Fractions 2, 9 and 13 previously giving MBC values of 6 μg/ml ($p<0.01$) were increased to values >50 μg/ml in the presence of DTT.

3.3.6 Antimicrobial activity of Defr1 1cys

A synthetic one cysteine dimeric analogue of Defr1 (Table 3.1) was created and analysed for antimicrobial function. Defr1 1cys was synthesised in an attempt to create a single defined species as Defr1 is a mixture. Defr1 1cys was synthesised, folded and was shown to be a homodimer by Albachem Ltd (personal communication Albachem Ltd). Defr1 1cys displayed potent antimicrobial activity against the panel of microorganisms, with the exception of *B.cenocepacia* (>100 μg/ml) (Figure 3.9 – 3.14).

Defr1 FRACTIONS	MBC (µg/ml)	Defr1 FRACTIONS	MBC (µg/ml)
1	6	**9**	6
2	6	**10**	6
3	6	**11**	6
4	6	**12**	6
5	6	**13**	6
6	6	**14**	6
7	6	**Defr1**	6
8	6		

Table 3.3 Defr1 HPLC Fractions

Defr1 HPLC fractions were tested for antimicrobial activity against *P.aeruginosa* PAO1. All fractions displayed potent antimicrobial activity. In addition Defr1 from the original preparation was tested as a comparison. Minimum Bactericidal Concentrations were presented as (µg/ml).

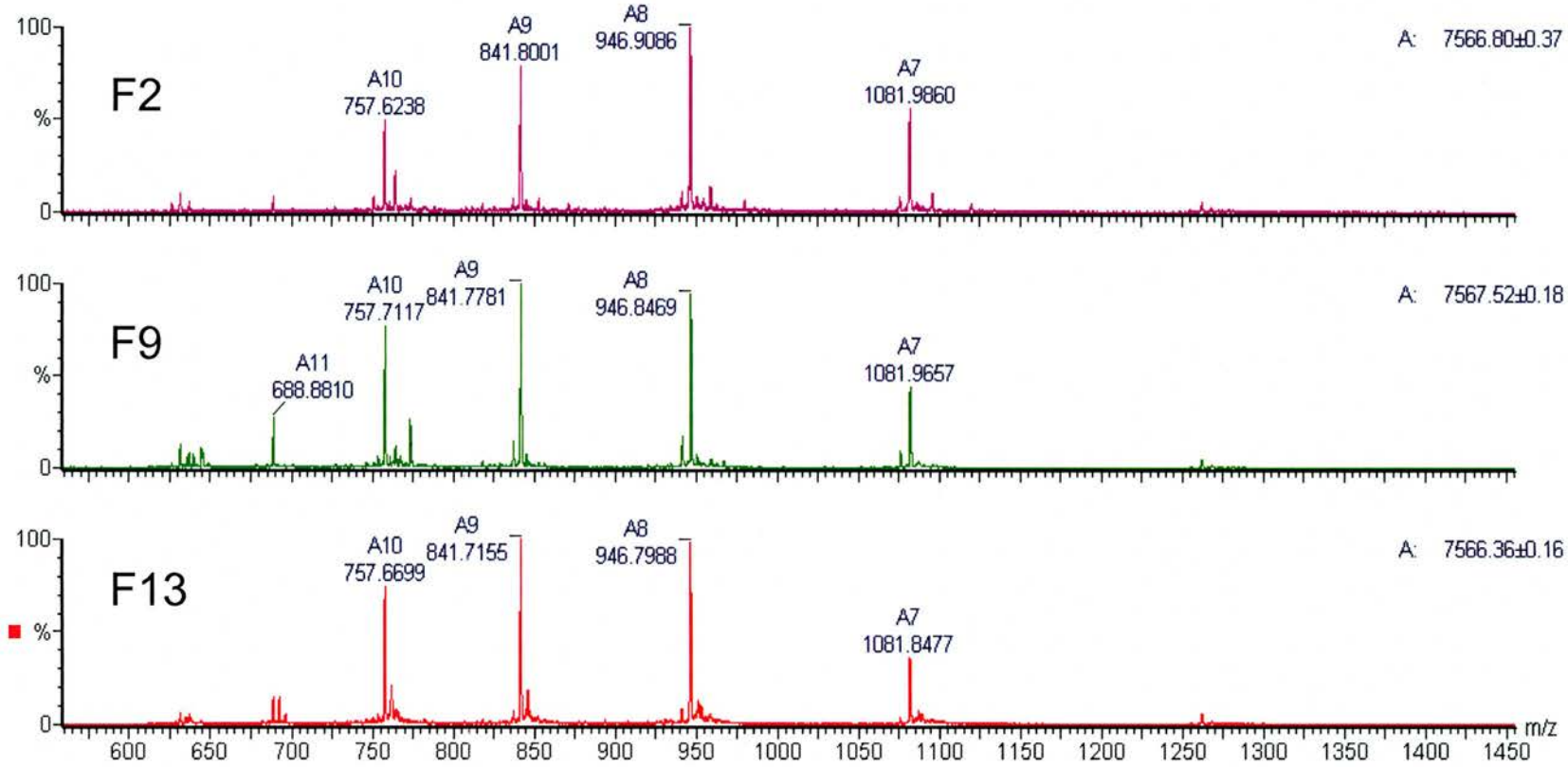


Figure 3.7 Mass spectrometry of Defr1 fractions 2, 9 and 13

The above figure displays the mass spectrometry of three Defr1 1 fractions (fractions 2, 9 and 13 – refer to Appendix 1). All three samples display similar mass spectrometry traces resulting in a mass of 7566 Da, 7567 Da and 7566 Da respectively. The expected mass of the dimeric Defr1 is 7566 Da (Campopiano *et al*., 2004) although no original stocks were available for testing alongside these fractions.

Performed by Bryan McCullough

PEPTIDE	MBC VALUES (µg/ml)	
	Non-reduced	Reduced
Fraction 2	6	>50
Fraction 9	6	>50
Fraction 13	6	>50
Defr1	6	>50

Table 3.4 MBC values of Defr1 fractions treated with DTT

Defr1 HPLC fractions 2, 9 and 13 were tested for antimicrobial properties against *P. aeruginosa* PAO1. Samples were tested in the presence (reduced) and in the absence (non-reduced) of DTT to determine if structural characteristics are implicated in the antimicrobial properties of Defr1. A sample of Defr1 from the original preparation was included.

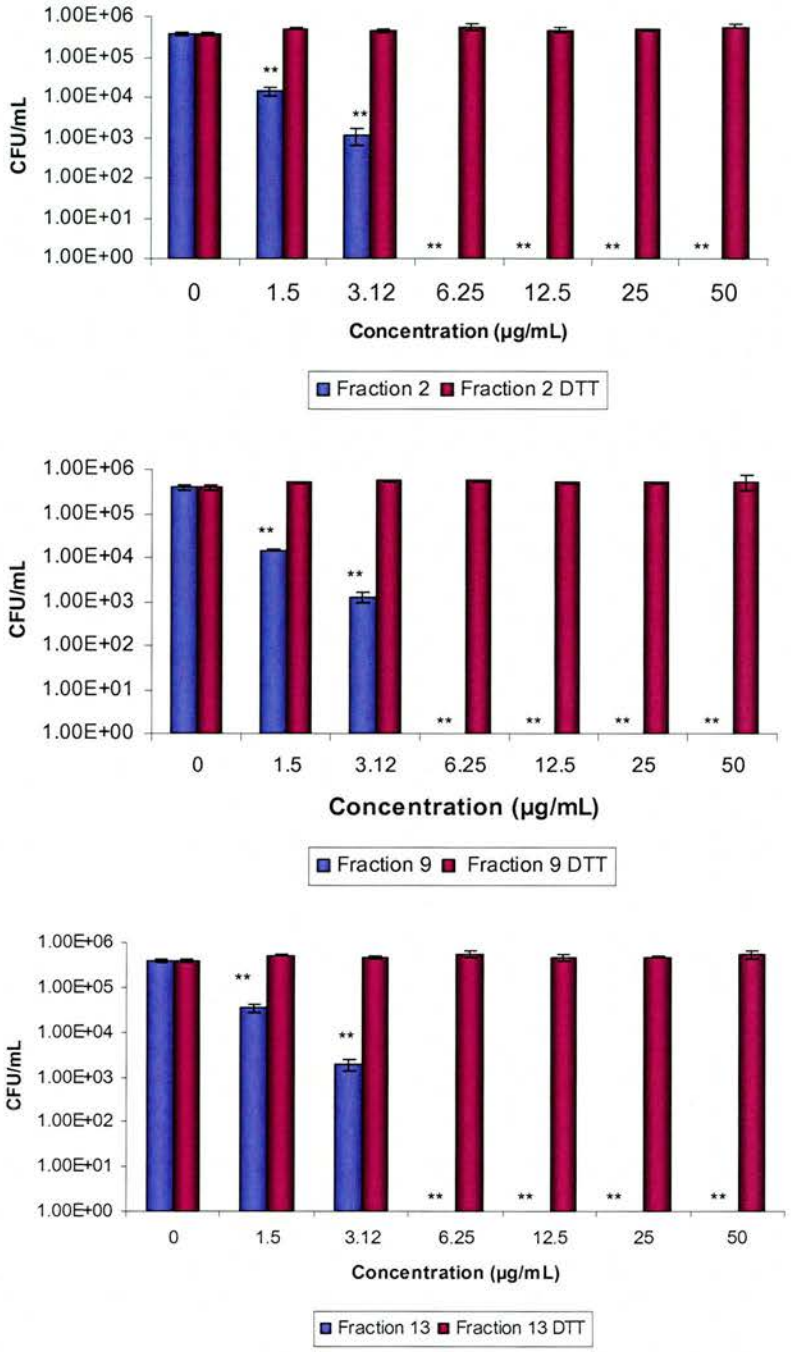
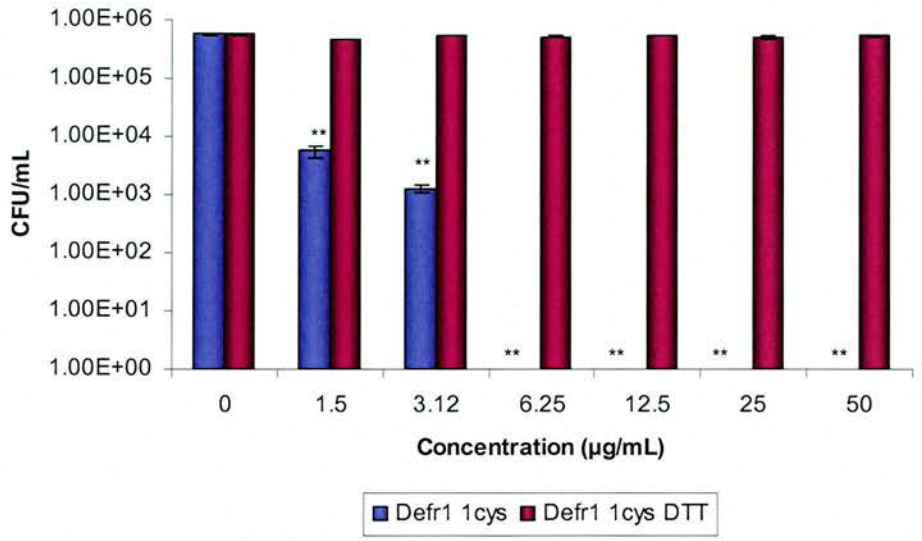


Figure 3.8 Antimicrobial activity of DTT treated Defr1 fractions
Graph A, B and C show the mean number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples (+/- 10mM DTT) against *P.aeruginosa* PAO1. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

A



B

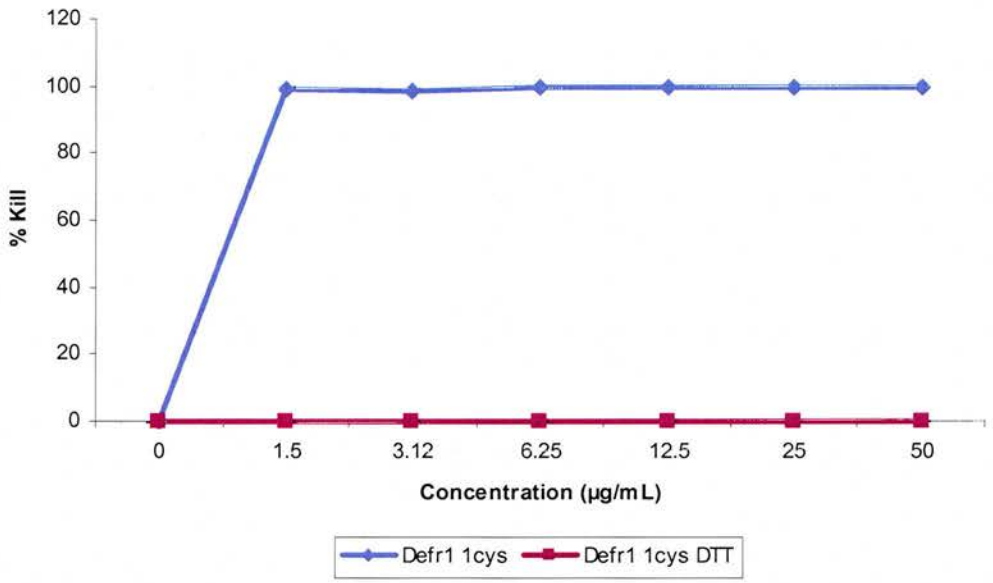
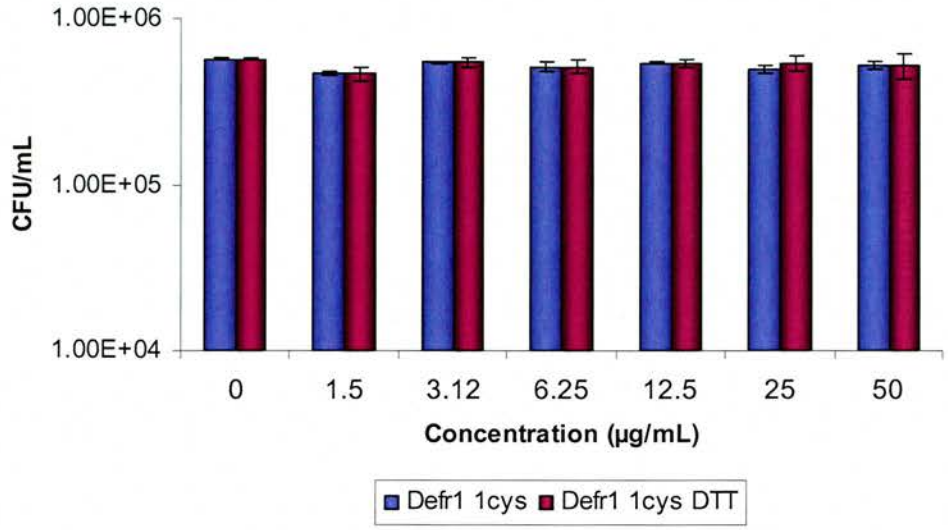


Figure 3.9 Antimicrobial activity of Defr1 1cys and Defr1 1cys DTT against *A.baumannii* ATCC 19606

Graph A shows the mean number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

A



B

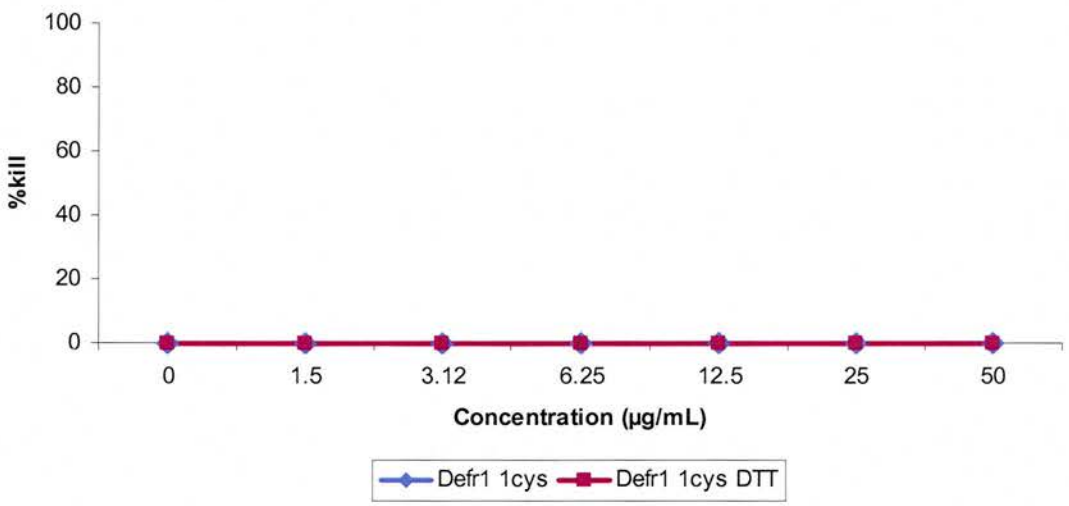
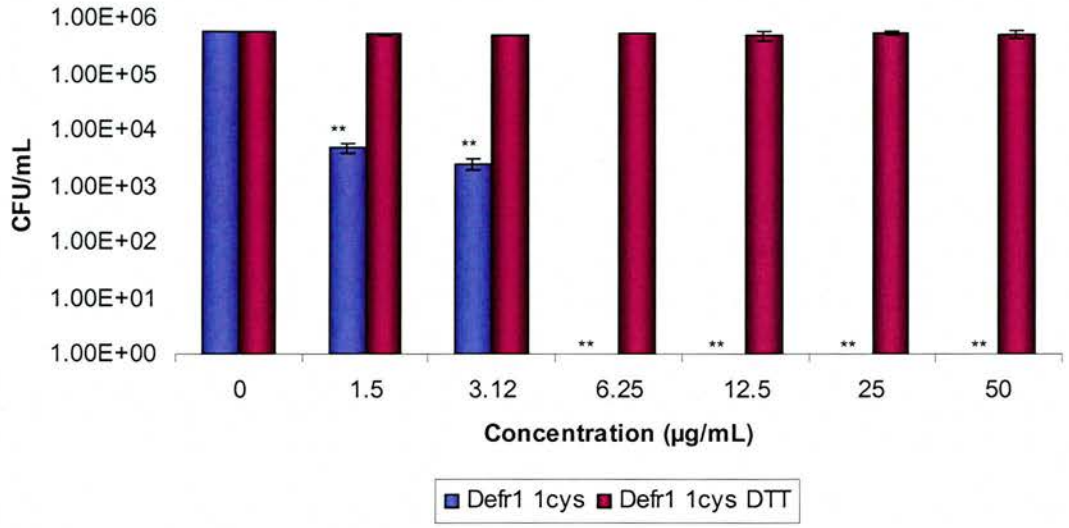


Figure 3.10 Antimicrobial activity of Defr1 1cys and Defr1 1cys DTT against *B.cenocepacia* J2315

Graph A shows the mean number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

A



B

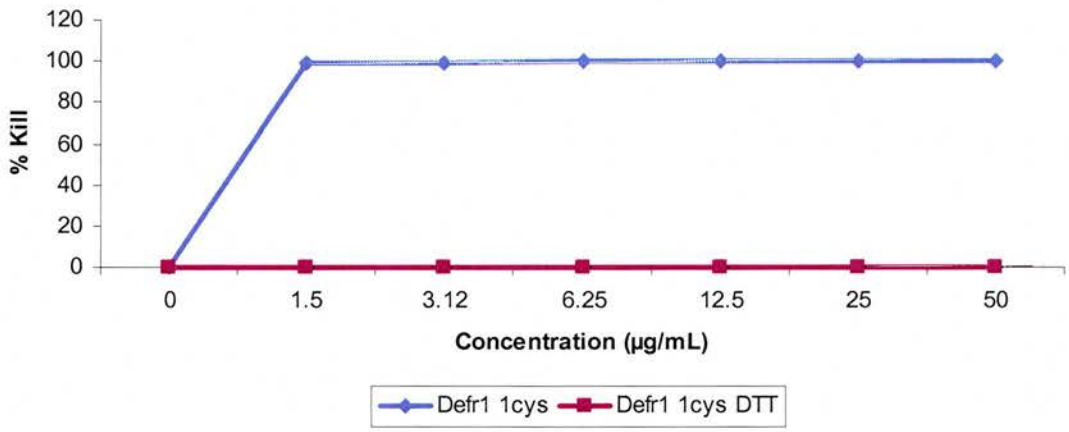
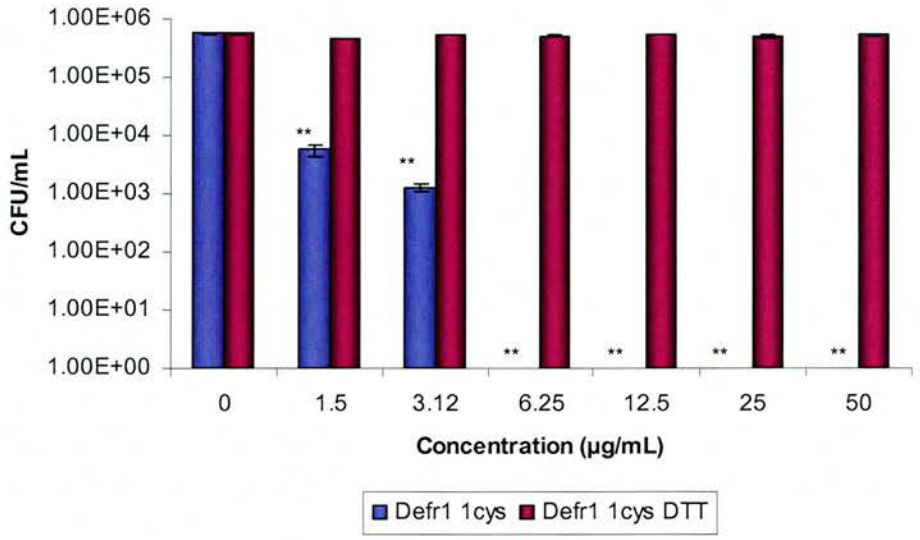


Figure 3.11 Antimicrobial activity of Defr1 1cys and Defr1 1cys DTT against *E.coli* ATCC 25922

Graph A shows the mean number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

A



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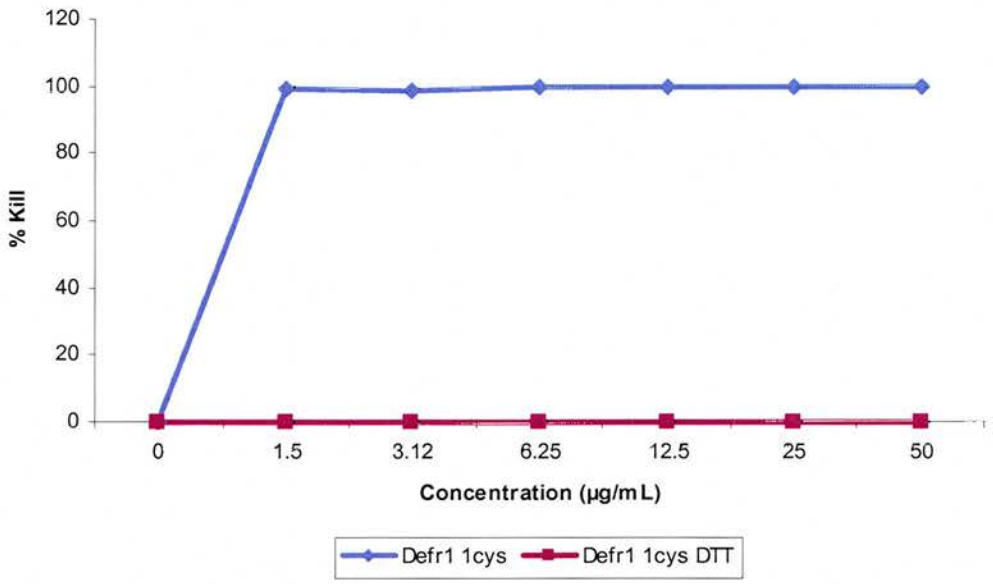
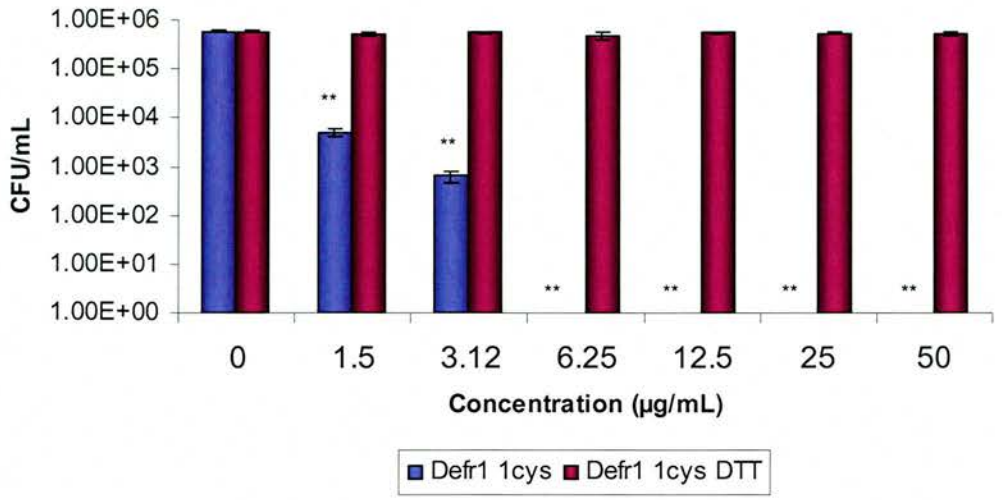


Figure 3.12 Antimicrobial activity of Defr1 1cys and Defr1 1cys DTT against *E.faecalis* ATCC 700802

Graph A shows the mean number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

A



B

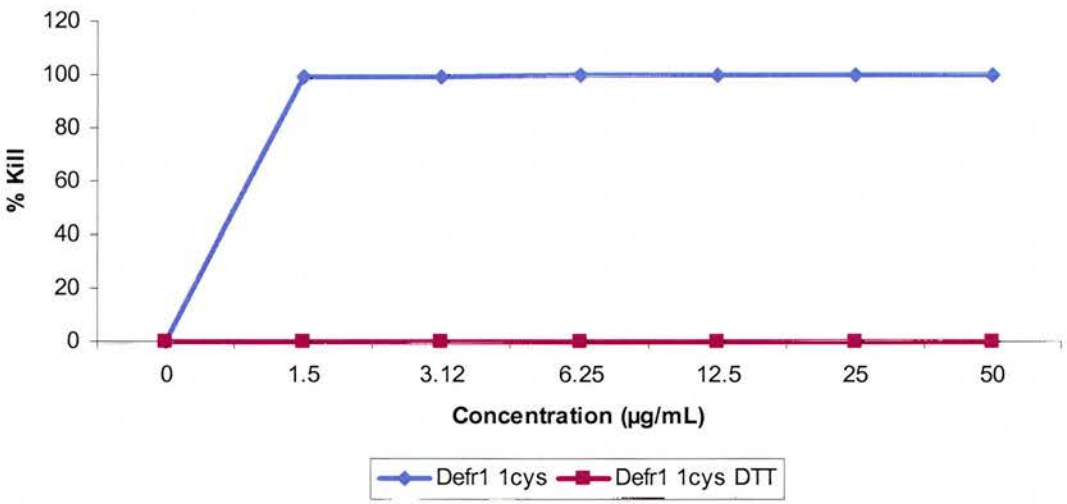
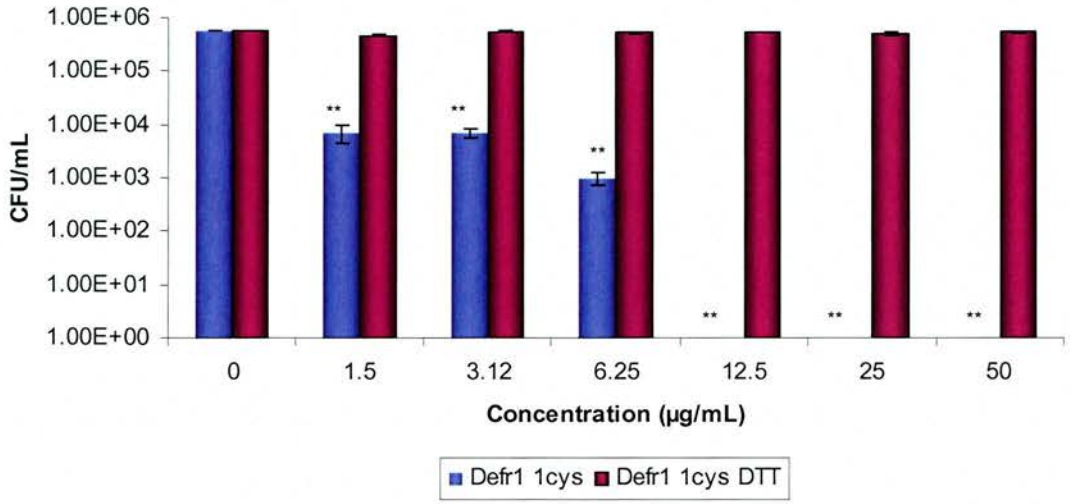


Figure 3.13 Antimicrobial activity of Defr1 1cys and Defr1 1cys DTT against *P.aeruginosa* PAO1

Graph A shows the mean number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

A



B

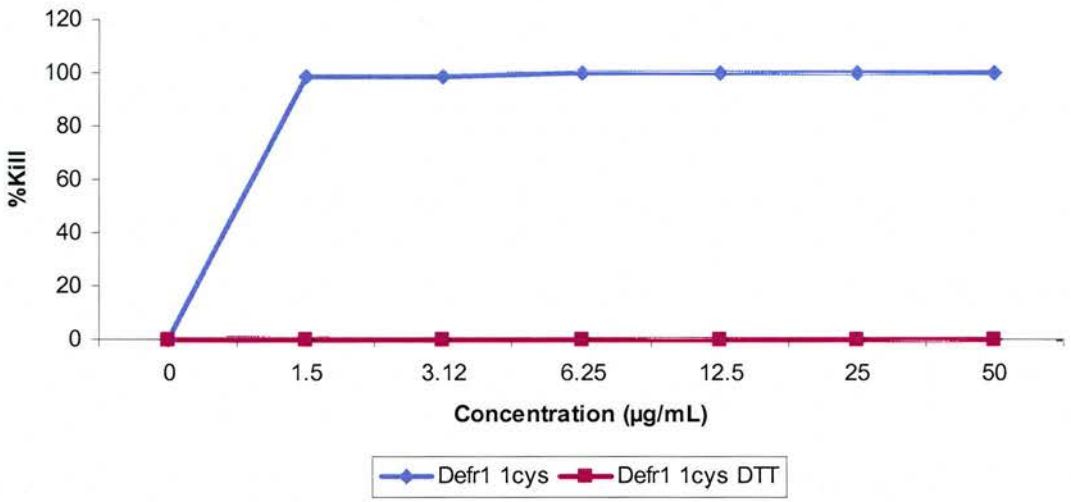


Figure 3.14 Antimicrobial activity of Defr1 1cys and Defr1 1cys DTT against *S.aureus* ATCC 25923

Graph A shows the mean number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

ORGANISM	MBC VALUE (µg/ml)	
	Defr1 1cys	Defr1 1cys reduced
A.baumannii ATCC 19606	6	>50
B.cenocepacia J2315	>50	>50
E.coli ATCC 25922	6.25	>50
E.faecalis ATCC 700802	6.25	>50
P.aeruginosa PAO1	6	>50
S.aureus ATCC 25923	12	>50

Table 3.5 Antimicrobial activity of Defr1 1cys and Defr1 1cys reduced

The antimicrobial activity of Defr1 1cys and Defr1 1cys reduced with DTT were tested against *P. aeruginosa* PAO1. Fractions treated with 10 mM DTT before performing antimicrobial analysis. Minimum Bactericidal concentrations were represented as µg/mL.

Defr1 1cys gave MBC values of 6 μg/ml and 12 μg/ml (Table 3.4). Further upon treatment with DTT the antimicrobial activity of Defr1 1cys is diminished from 6-12 μg/ml to >50 μg/ml.

3.3.7 Mass spectrometry of Defr1 1cys

Defr1 1cys was analysed by Q-ToF MS by Bryan McCullough (Figure 3.15). Defr1 1 was analysed at 10 μM 1:1 MeOH/DH_20 and displayed dimeric properties.

3.3.8 Gel electrophoresis of Defr1and Defr1 1cys

Characterisation of the Defr1 peptide on a 16% tricine gel revealed that the preparation consists of a dominant upper band at 7kDa and a faint lower band at 4kDa (Figure 3.6). However mass spectrometry showed it exists as a covalent dimer (Campopiano *et al.*, 2004). Reduction of Defr1 with 10 mM DTT results in a shift of the migration of the peptide to a stronger band at around 4kDa. Defr1 1cys displayed similar mobility on the gel with a band around 7kDa for the oxidised Defr1 1cys and upon reduction with DTT a shift is observed to around 4kDa (Figure 3.16)

3.3.9 Chemoattractant analysis of Defr1, 5YC and Defr1 1cys

Defr1, Defr1 1cys and 5YC were evaluated for chemotactic activity against neutrophils, CD4+ T cells (human & mouse) and CCR6 expressing HEK293 cells (Figures 3.17-3.20). The Defr1 parent molecule displays chemotactic activity for CD4+ T cells, human and mouse, with an optimum concentration of 10 ng/ml (Table 3.6). Defr1 1cys and 5YC also induce migration with optimum concentrations at 100 ng/ml. The single cysteine dimer analogue displays ten fold greater activity in comparison with the parent Defr1. No migration was observed with any of the peptides against murine neutrophils. In addition no migration was observed with CCR6 expressing HEK293 cells.

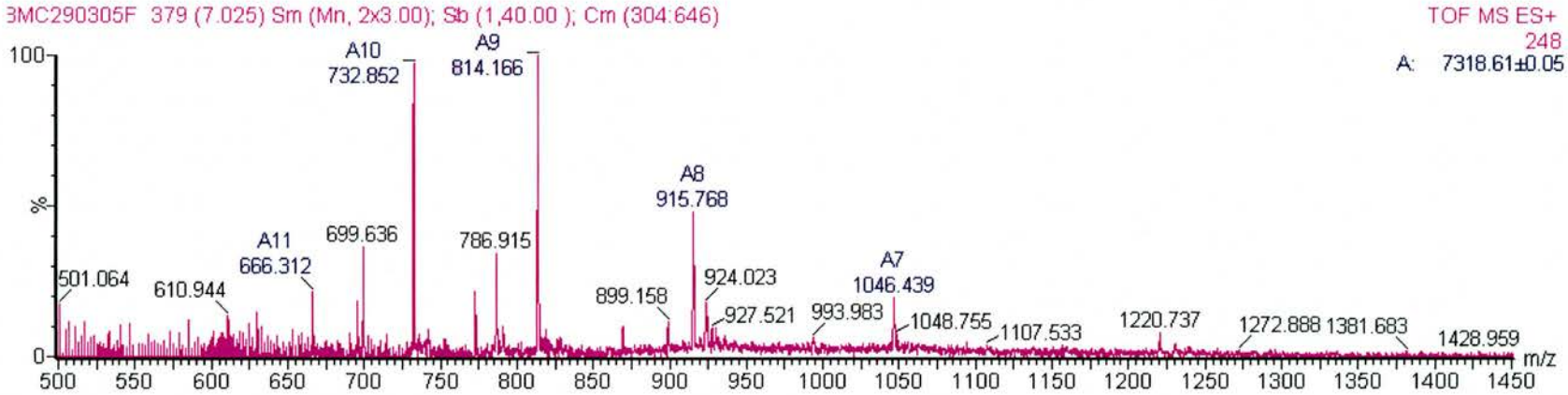


Figure 3.15 Mass spectrometry of Defr1 1cys

The above mass spectrometry displays the peptide Defr1 1cys. This mass spectrometry trace predicts a mass of 7318 Da for the one cysteine variant. The original Defr1 displays a mass of 7566 Da.

Performed by Bryan McCullough

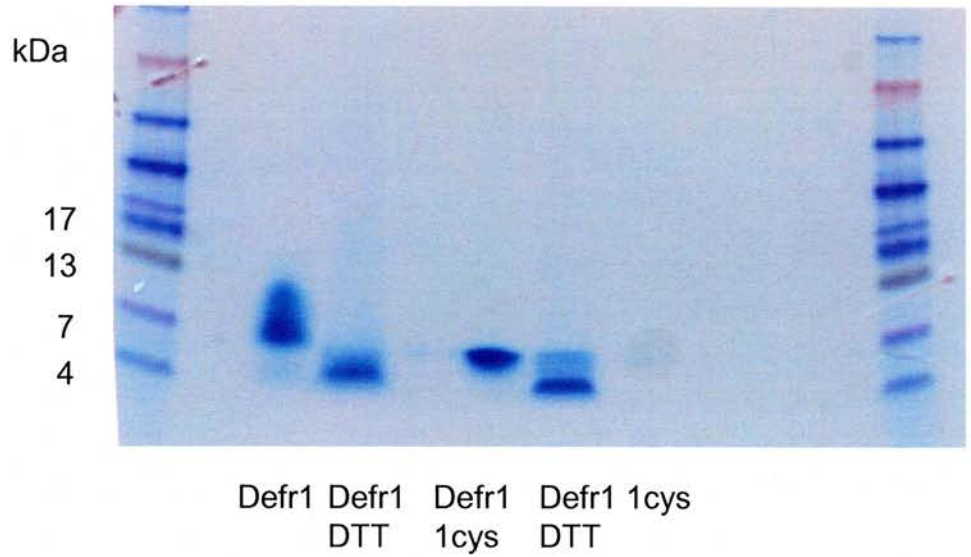
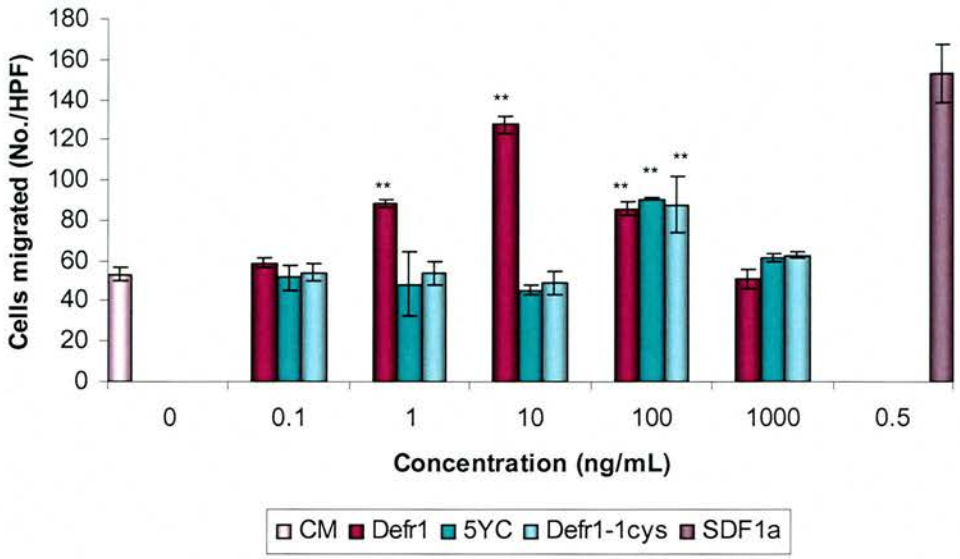


Figure 3.16 Gel electrophoresis of Defr1 and Defr1 1cys

Both Defr1 and Defr1 1cys were run on a 16% tricine gel. Samples were oxidised and reduced (with 10 mM DTT) prior to loading on the gel.. The addition of 10mM DTT displays a slight shift in both Defr1 and Defr1 1cys when compared to the oxidised samples. (Defr1 was from previous Albachem stocks.

A



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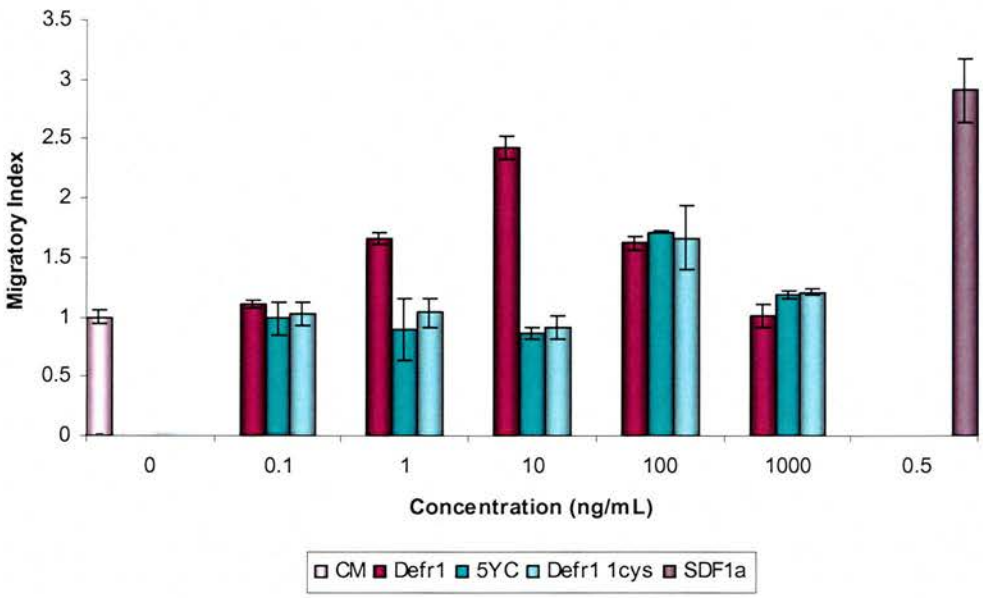
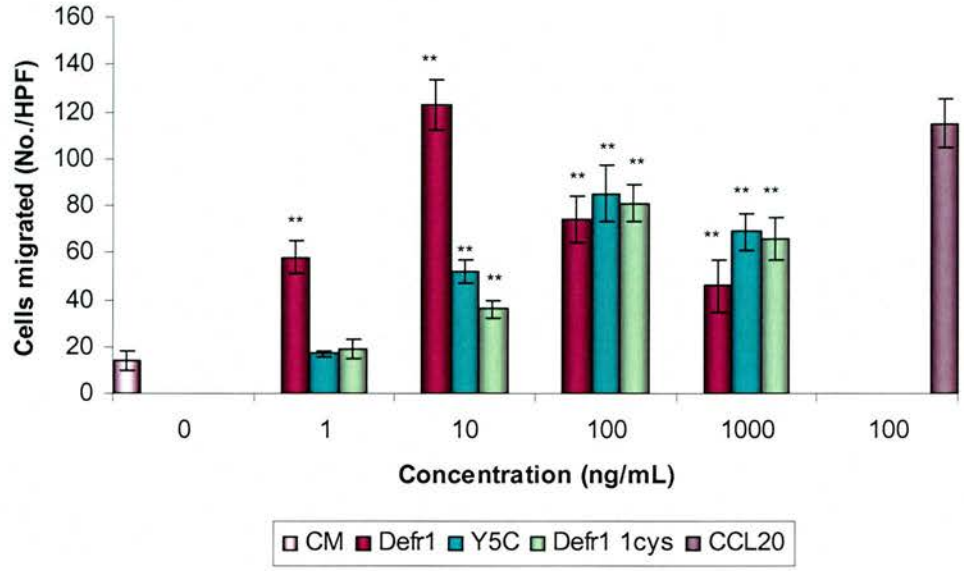


Figure 3.17 Migration of mouse CD4+ T cells in response to Defr1 Defr1 1cys and 5YC

Graph A shows the mean number of cells migrated per random field of view. Graph B shown the migratory index, the number of cells migrated in each sample divided by the number of migrated cells in the media alone sample. * represents $p< 0.05$, ** $p<0.01$. Error bars represent the standard error of all nine replicate counts. Experiments were repeated three times and the above is a representative experiment (n=3).

A



B

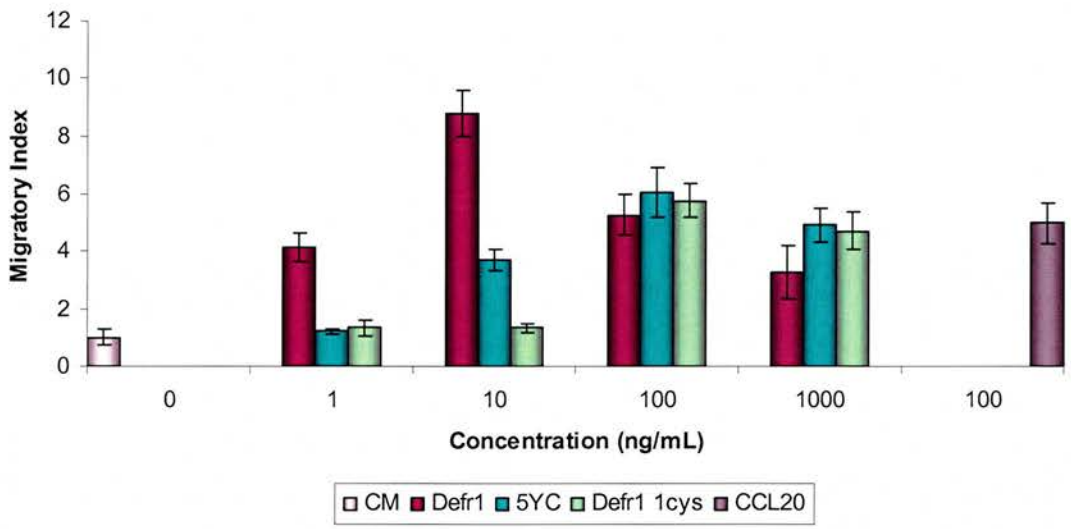
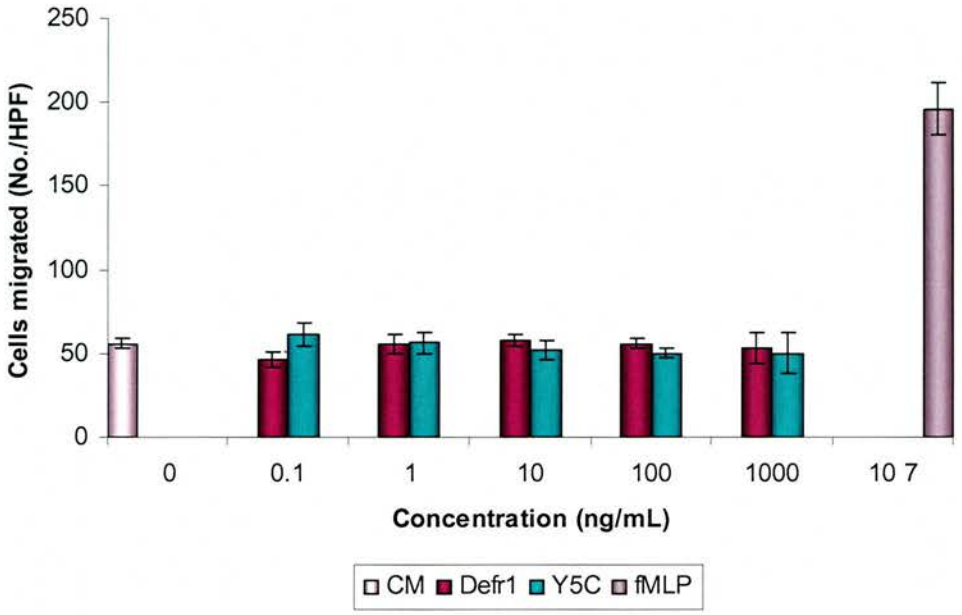


Figure 3.18 Migration of human CD4+ T cells in response to Defr1 Defr1 1cys and 5YC

Graph A shows the mean number of cells migrated per random field of view. Graph B shown the migratory index, the number of cells migrated in each sample divided by the number of migrated cells in the media alone sample. * represents $p< 0.05$, ** $p<0.01$. Error bars represent the standard error of all nine replicate counts. Experiments were repeated three times and the above is a representative experiment (n=3).



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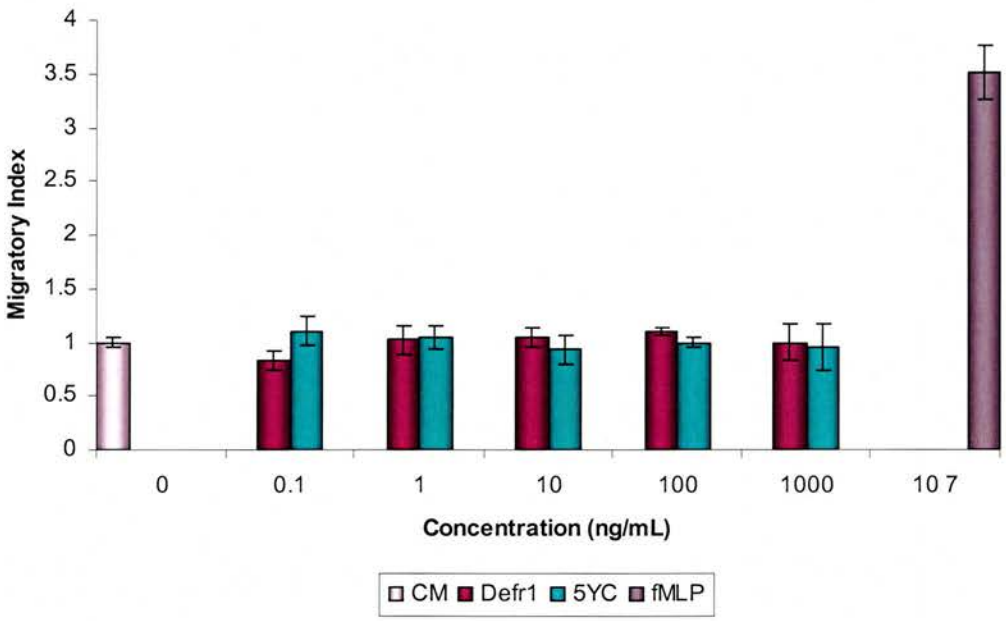
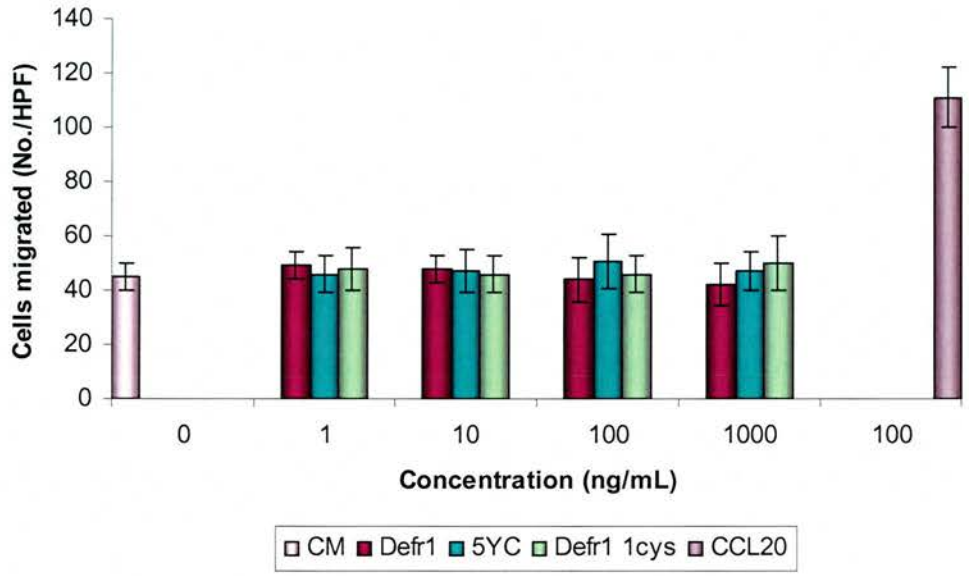


Figure 3.19 Migration of mouse neutrophils in response to Defr1 Defr1 1cys and 5YC
Graph A shows the mean number of cells migrated per random field of view. Graph B shown the migratory index, the number of cells migrated in each sample divided by the number of migrated cells in the media alone sample. * represents $p< 0.05$, ** $p<0.01$. Error bars represent the standard error of all nine replicate counts. Experiments were repeated three times and the above is a representative experiment (n=3).

A



B

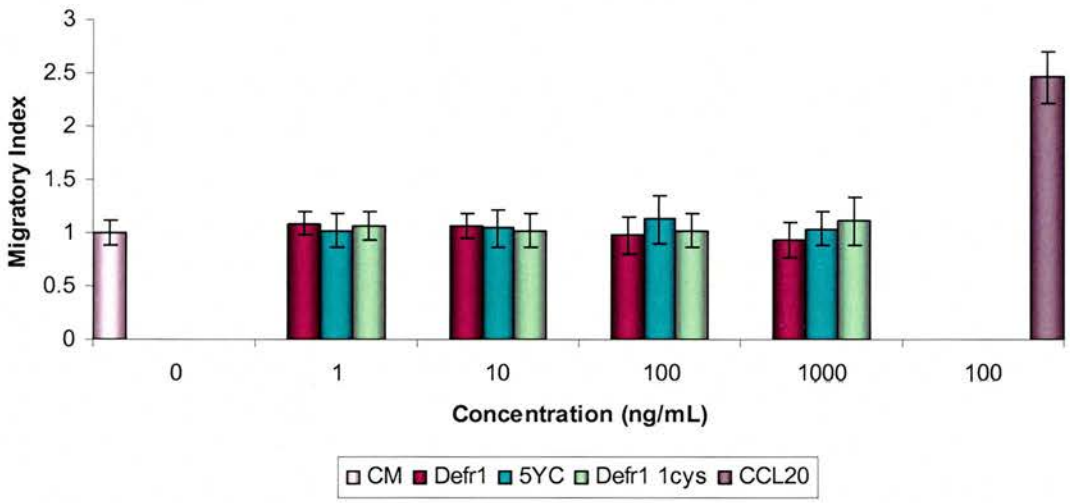


Figure 3.20 Migration of HEK 293 CCR6 cells in response to Defr1 Defr1 1cys and 5YC

Graph A shows the mean number of cells migrated per random field of view. Graph B shown the migratory index, the number of cells migrated in each sample divided by the number of migrated cells in the media alone sample. * represents $p< 0.05$, ** $p<0.01$. Error bars represent the standard error of all nine replicate counts. Experiments were repeated three times and the above is a representative experiment (n=3).

CELL TYPE	Defr1	5YC	Defr1 1cys
CD4 T cells (human)	10	100	100
Neutrophils (mouse)	no substantial migration	no substantial migration	-
CD4 T cell (mouse)	10	100	100
HEK293	no substantial migration	no substantial migration	no substantial migration
HEK293 CCR6	no substantial migration	no substantial migration	no substantial migration

Table 3.6 Chemotactic analysis of Defr1, 5YC and Defr1 1cys

Shown is the optimal concentration (ng/ml) of Defr1, Defr1 1cys and 5YC that induced the maximal migration of CD4 T cells (human), neutrophils (mouse), CD4 T cells (mouse), HEK293 and HEK293 CCR6.
Not tested represented as -
Highest test concentration 1000 ng/ml

3.4 DISCUSSION

Through evaluation of Defr1, 5YC and Defr1 1cys this study has shown the following:

- That Defr1 displays antimicrobial activity against a panel of microorganisms, including multi-resistant isolates.
- That Defr1 HPLC fractions are a mixture similar to that of the original Defr1.
- That Defr1 HPLC fractions are similar by mass spectrometry and display identical antimicrobial values against *P.aeruginosa*.
- That the synthetic dimeric analogue Defr1 1cys dimer displays antimicrobial properties comparable with that of Defr1 and upon reduction with DTT the activity diminishes.
- That Defr1, Defr1 1cys and 5YC display chemoattractant properties for CD4+ T cells
- That Defr1, Defr1 1cys and 5YC do not chemoattract using CCR6 expressing HEK293, the proposed receptor for β-defensin chemotaxis.

3.4.1 Antimicrobial analysis of Defr1 and 5YC against a panel of multiresistant organisms

Defr1 and 5YC were both evaluated for antimicrobial activity against a panel of organisms, including some multi-resistant, problematic isolates. Organisms including *Acinetobacter*, *Burkholderia*, *Ralstonia* and *Stenotrophomonas* specieswere tested. The gram negative *Acinetobacter baumannii* is generally considered non pathogenic but is frequently isolated in nosocomial infections. *A. baumannii* causes mild to sever illness but can be fatal due to its frequent resistance to establish antimicrobials (Joly-Guillou, *et al.*, 2005). *B. cenocepacia* are important opportunistic pathogens regarding cystic fibrosis (Mahenthiralingam *et al.*, 2005). Displaying natural resistance to many antimicrobials *B. cenocepacia* causes variable lung infections resulting in chronic infections often requiring isolation. *Ralstonia* species are generally considered to be of

little clinical significance although reports have highlighted their role in bacteraemia (Ryan *et al.*, 2006). Invasive and severe infections have been reported with this persistent nosocomial organism. Although uncommon *Stenotrophomonas maltophilia* is difficult to treat due to its resistance to broad spectrum antibiotics. *S. maltophilia* is commonly associated with infections of the tracheal tubes (McGowan *et al.*, 2006).

Both Defr1 and 5YC contain 34 residues and display an overall net charge of +6 (Table 3.1). The only notable difference is the replacement of a tyrosine residue with a cysteine residue reinstating the β-defensins cysteine backbone. Both peptides form dimers (Campopiano *et al.*, 2004) yet give different antimicrobial profiles against the organisms tested. Defr1 displays potent antimicrobial activity against a broad spectrum of pathogens. This is the first documented study involving an extensive panel of multi-resistant nosocomial isolates tested against a β-defensin. A previous study regarding HBD3 (Maisetta *et al.*, 2006) involved analysing the antimicrobial activity of six different isolates from *S.aureus*, *E.faecium, P.aeruginosa, S.maltophilia* and *A.baumannii*. The *A.baumannii* isolates included bacterial strains that were resistant to one or two classes of antimicrobials yet still gave an MBC value of 4 μg/ml (Maisetta *et al.*, 2006). Only the *B.cenocepcia* strains (Table 3.2) showed high levels of resistance to Defr1 (MBC>100 μg/ml), a result which is in agreement with the characteristic resistance of these bacteria to conventional antibiotics and antimicrobial peptides (Sahly *et al.*, 2003). Previously (Morrison et al., 2002b) reported that the bactericidal activity of Defr1 against *B.cenocepacia* J2315 gave only 40% killing when tested at 50 μg/ml. Regarding 5YC this peptide displayed antimicrobial activity against a few isolates but this required a higher concentration to observe killing than that with Defr1. The majority of values observed were greater than 100 μg/ml. Previously, the differences in antimicrobial activity between Defr1 and 5YC were attributed to their structure (Campopiano *et al.*, 2004). 5YC forms a noncovalently bound dimer in solution and retains the disulfide connectivity typical of that within the β-defensins. Defr1 reveals a covalent dimer containing an intermolecular disulfide bond and is a complex mixture of dimer isoforms, varying in intra and inter-molecular disulfide connectivities. Upon reduction and subsequently a loss of structure, the antimicrobial activity of Defr1

diminishes. Interestingly NMR studies of Defb8 / mBD-8 (Bauer et al., 2001) revealed that this was a monomer, unlike that of the dimer evident with Defr1. mBD- 8 has been synthesised (Kluver et al., 2002) but no functional studies of the peptide have been published.

3.4.2 Defr1 HPLC Fractions

HPLC was performed by Albachem Ltd and fractions collected, lyophilised and resuspended in 0.01 % acetic acid. Gel electrophoresis and antimicrobial assays of the fractions were performed. Antimicrobial analysis revealed that all the fractions and the parent Defr1 displayed activity at 6 μg/ml (Table 3.3). Gel electrophoresis of the fractions revealed different intensities on the 16% tricine gel implying different structures. Fractions 2, 9 and 13 were selected due to their noticeable differences on the gel and studied by mass spectrometry (Figure 3.6 & 3.7). Despite the apparent mobility differences the structure of the three fragments was consistent. Upon reduction of the fractions with DTT the antimicrobial activity was diminished (Table 3.4) and mass spectrometry confirmed the loss of dimeric structure. The revelation that Defr1 contains a mixture of different isoforms is not an unusual discovery with the β-defensins. Previous work on synthetic HBD3 led to the discovery that it did not fold into a single isoform with the typical β-defensin disulfide connectivity in vitro (Wu *et al.*, 2003). Instead, at least seven topologically distinct isoforms were isolated with unusual intramolecular connectivities, all of which displayed similar antimicrobial activity.

3.4.3 Antimicrobial analysis of Defr1 1cys

In an attempt to confirm the importance of a dimeric-activity relationship, a one cysteine variant (Table 3.1) was synthesised. Defr1 1 cys was tested antimicrobially against both Gram positive and Gram negative organisms (Figure 3.9-3.14) and displayed results similar to that of the original Defr1 peptide. Defr1 1 cys forms a covalent dimer and again similar to that of the original Defr1the antimicrobial activity is diminished and the dimeric structure is lost upon reduction with DTT. The presence of DTT does not alter bacterial viability (personal communication Ross Langley). These

results strongly suggest that it is the covalent dimerisation of Defr1 that makes it a powerful antimicrobial and the synthesis of Defr1 1 cys confirms this. Synthetic sequences based on the Defr1 β-defensin sequence are antimicrobially potent even in the absence of the canonical cysteine motif. This has been confirmed with HBD3 and the synthesis of the peptide with the cysteines replaces with α-aminobutyric acid (Hoover *et al.*, 2003). The peptide still retains its antimicrobial activity.

3.4.4 Chemotactic analysis of Defr1, 5YC and Defr1 1cys

Many β-defensins can act as chemoattractants for cells involved in host defense. Defr1, 5YC and Defr1 1cys were evaluated against a panel of different cell types. Chemotaxis was observed using CD4+ T cells. This is not an unusual observation as various defensins have displayed the ability to chemoattract for these cells. However no migration was observed with the mouse neutrophils. These cells have not been reported to display chemoattractant properties with the β-defensins but they do display migration with other antimicrobial peptides, LL-37 (Yang *et al.*, 2001) with an optimum concentration of 10^{-5}M and PR-39 (Huang *et al.*, 1997) at a peak concentration of 0.5-2 μM.

An interesting property to note is that all three defensins tested here contain varying numbers of cysteine residues yet still retain chemoattractant properties. Previous studies with the β-defensins show that the cysteine residues are essential for chemoattractant activity. Studies with HBD3 whereby the cysteine residues were replaced with α-aminobutyric acid (Wu *et al.*, 2003) gave no migration for both monocytes and HEK293 cells expressing CCR6 at concentrations greater than 10000 ng/ml. Defr1 contains 5 cysteines, 5YC contains 6 and Defr1 1cys contains 1 cysteine residues and all three display chemoattractant properties against certain cell types. Also Defr1 is a mixture of dimeric isoforms yet 5YC conforms to the β-defensin connectivity and both induce migration although Defr1 appears to induce a higher number of cells at a value 10-fold less than that of 5YC and Defr1 1cys.

In order to assess if Defr1, 5YC and Defr1 1 cys utilise the chemokine receptor 6 (CCR6) in the migration observed via chemotaxis, HEK293 cells expressing CCR6 were

used. No migration was observed with Defr1, 5YC or Defr1 1cys suggesting these peptides do not use CCR6 within this chemotaxis assay. Previous analysis with the β-defensins has displayed the importance of CCR6 in migration (Yang *et al.*, 1999) with this being highlighted as the receptor involved. However other studies have suggested this is not the only receptor. HBD3 displays chemoattractant properties towards monocytes, which do not express functional CCR6 therefore suggesting an additional receptor is involved (Wu *et al.*, 2003). No studies have currently revealed what this receptor is. Other antimicrobial peptides utilise different receptors to that of the β-defensins. LL-37 has been shown to act through the Formyl Peptide Receptor-Like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes and T cells (Yang *et al.*, 2000).

In summary, these results highlight that Defr1 displays potent antimicrobial activity against an array of pathogens. Being a mixture of different isoforms Defr1 HPLC fractions displayed similar antimicrobial properties and by mass spectrometry were identical. The synthesis of Defr1 1cys emphasised that the dimeric structure was essential for activity with this peptide and upon reduction the activity was lost. Migration in response to Defr1, 5YC and Defr1 1cys was achieved despite a varying number of cysteine residues. Importantly these peptides were shown not to induce migration of HEK293 cells expressing CCR6. To date, this is the first example of a β-defensin failing to induce migration of HEK293 CCR6 cells.

CHAPTER 4:

Defb14 THE MURINE ORTHOLOGUE OF HUMAN β-DEFENSIN 3

4.1 INTRODUCTION

Evolutionary analysis of the β-defensin superfamily demonstrates that the murine orthologue of the widely studied human β-defensin 3 (HBD3) is Defb14 (Semple *et al.*, 2005). This gene *Defb14*, encodes a predicted peptide which is 68% identical to that of HBD3 and notably displays only four amino acid changes that are not conserved or semi-conserved. The second exon sequence of *Defb14* was first discovered by a genomic approach (Schutte *et al.*, 2002).

Human β-defensin 3 (DEFB103) is a member of the β-defensin multigene family located in the major defensin locus on the human chromosome 8 (Garcia *et al.*, 2001a, Harder *et al.*, 2001). HBD3 was initially identified in lesional psoriatic scales and was subsequently cloned from foreskin derived keratinocytes and lung epithelial cells (Harder *et al.*, 2001). In additional studies HBD3 was cloned based on bioinformatics and functional genomic analysis (Jia *et al.*, 2001, Garcia *et al.*, 2001a).

The isolation and purification of HBD3 from lesional psoriatic scales, keratinocytes and lung epithelia revealed these as an effective source for the peptide. Expression of HBD3 mRNA was detected predominantly in epithelial tissues but was also found in non-epithelial sources. The skin, tonsils, gingival keratinocytes, skeletal muscle, adult heart, testis and oesophagus are the major tissues expressing HBD3 (Harder *et al.*, 2001, Jia *et al.*, 2000, Garcia *et al.*, 2001a). The induction of HBD3 has become evident in HaCaT keratinocytes with the exposure to IFN-γ giving rise to a 136-fold increase in transcripts (Garcia *et al.*, 2001a, Abiko *et al.*, 2003). HBD3 has also been show to be induced by bacterial stimulus. In a study with *P.aeruginosa* clinical isolates and human primary keratinocytes, HBD3 was shown to be strongly expressed (Harder *et al.*, 2001).

The mature form of the HBD3 peptide consists of 45 amino acids and is a highly basic antimicrobial peptide. Antimicrobial activity has been detected in synthetic, recombinant and naturally occurring peptides. HBD3 displays potent broad spectrum activity against Gram-negative bacteria, Gram-positive bacteria and fungi (Harder *et al.*, 2001, Garcia *et al.*, 2001a, Sahly *et al.*, 2003). Antimicrobial activity has been detected against a wide range of pathogens. Minimal bactericidal values against *S.aureus* ATCC

6538 give 1.5-6.3 μg/ml (Harder *et al.*, 2001), *S. epidermidis* 1.6 μg/ml, *E. coli* 0.4 μg/ml, *Acinetobacter sp* 0.2-1.6 μg/ml (Sahly *et al.*, 2003) and *B. cenocepacia* ATCC 17770 6.6μg/ml (Garcia *et al.*, 2001). Subsequent studies regarding *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis* and *B. multivorans* have displayed high resistance (MBC value >100 μg/ml) towards human β-defensin 3 (Sahly *et al.*, 2003). Multidrug-resistant nosocomial strains have been evaluated against HBD3 giving MBC values of 4-8 μg/ml against *S. maltophilia*, 4-8 μg/ml against *S. aureus* and 8 μg/ml against *E. faecium* (Maisetta *et al.*, 2006). Recently extended spectrum β –lactamase (ESBL)-producing *Klebsiella* strains have been tested. Generally the strains show high susceptibility to HBD3 with MBC values in the range of 3.12 - 25 μg/ml (Sahly *et al.*, 2006). Further studies have been conducted using HBD3 in combination with other antimicrobial agents, lysozyme, metronidazole, amoxicillin and chlorohexidine against oral pathogens. In combination the bactericidal activity of HBD3 was shown to be enhanced (Maisetta *et al.*, 2003). HBD3 has also demonstrated salt insensitive antimicrobial activity with *S. aureus* ATCC 6538 (Harder *et al.*, 2001). Against *S. aureus* HBD3 has been shown to retain 90% killing at 250mM NaCl concentration.

The mechanism of HBD3 antimicrobial activity is not yet known. Like other β-defensins, it is believed that the antimicrobial action of HBD3 is based on electrostatic interactions although this mechanism is poorly understood. The membrane activity of HBD3 has been investigated using membrane lipid compositions, sensitive LPS strains of *S. enterica* sv Minnesota R595, *E. coli* F515 and resistant *P. mirabilis* R45 isolates (Bohling *et al.*, 2006). This study has shown a clear correlation between the biological activity of HBD3 and the interaction with the membrane lipid composition particularly with the outer membrane lipids from sensitive bacterial strains. Ultrastructural studies have been performed to develop an insight into the mechanisms by which HBD3 kills *S. aureus*. Morphological changes were examined using transmission electron microscopy and revealed signs of perforation of the peripheral cell wall, liberation of the plasma membrane and bacteriolysis after two hours (Harder *et al.*, 2001). Regarding mammalian cell membranes numerous studies have been performed discussing the hemolytic and cytotoxic properties of HBD3 (Kluver *et al.*, 2005, Harder *et al* 2001).

HBD3 was assessed for cytotoxic activity against human erythrocytes with no significant hemolysis detected except when associated with high concentrations of HBD3 (500μg/mL)(Harder *et al.*, 2001).

The β-defensins have been shown to maintain a role in both the innate and adaptive immune response. Many defensins including HBD3 act as chemoattractants at nanomolar concentrations with activity against a variety of cell types. HBD3 has induced migration of monocytes with a maximal response at 100ng/ml (Wu *et al.*, 2003) although no migration was evident with neutrophils (Garcia *et al.*, 2001a). Migration has been observed with $CD4^+$ T cells and immature dendritic cells but not shown with mature dendritic cells. CCR6 appears to be the chemokine receptor that the β-defensins seem to be acting through (Yang *et al.*, 1999). Migration was observed with HEK293 cells transfected with CCR6 at an optimal concentration of 10ng/ml. HBD3 has been shown to be active against both the CCR6 chemokine receptor and another unidentified receptor present on monocytes (Wu *et al.*, 2003).

Structure activity relationships associated with HBD3 have been studied extensively. Structural characteristics between several β-defensins have confirmed the disulfide connectivity observed within the family is (Cys1-Cys5, Cys2-Cys4, Cys3-Cys6) (Ganz *et al.*, 1999). Chemically synthesised analogues with predefined sulfide connectivities have been created and tested for their antimicrobial and chemoattractant properties. All analogues exhibited similar antimicrobial characteristics whereas various chemoattractant properties were observed. Included in this analysis was native HBD3 which *in vitro* does not fold preferentially into the described native conformation (Wu *et al.*, 2003). Further analysis involved replacement of all the cysteine residues with α-aminobutyric acid which rendered the peptide inactive chemotactically and yet enhanced its antimicrobial properties (1 μg/ml against *S. aureus*) (Wu *et al.*, 2003, Hoover *et al.*, 2003). Native disulfide linkages and non-native linkages, varying peptide sequence lengths, hydrophobicities and net charges have been evaluated in an attempt to elucidate the functional requirements (Wu *et al.*, 2003, Hoover *et al.*, 2003, Kluver *et al.*, 2005).

This chapter describes the first documented study involving the characterisation of Defb14. Synthetic peptide preparations were evaluated in numerous experiments for

functional analysis. A synthetic analogue, Defb14 1cys was analysed to elucidate a connection between structural differences and functionality with that of the original Defb14 peptide preparation. In addition Defb14 was synthesised whereby the cysteine residues were replaced with alanine residues. This peptide was analysed for antimicrobial and chemoattractant properties.

4.2 AIMS

The aims of this chapter were to:

1 Characterise a synthetic preparation of Defb14 the murine orthologue of HBD3

2 Evaluate Defb14 1cys, a synthetic dimeric analogue of Defb14

3 Examine both Defb14 and Defb14 1cys for relationships in structure and function

4.3 RESULTS

A synthetic preparation of Defb14 was examined in order to characterise the antimicrobial and chemoattractant properties. To gain an understanding into the structural requirements for functionality a further peptide, dimeric Defb14 1cys was characterised. In addition to structure and function, the ability of these peptides to lyse human erythrocytes was also evaluated.

4.3.1 Defb14 is the orthologue to DEFB103 (HBD3)

Defb14 is 68% identical to that of the HBD3 primary sequence (Table 4.1). Only 4 amino acid changes have been noted that are not conserved or semi-conserved within the sequence. Evolutionary analysis of the β-defensin family demonstrates that *DEFB103* and *Defb14* are orthologues and that there is a complexity of selective pressures acting on this gene family (Semple *et al.*, 2005). Of the four non-conservative changes, one lies between cysteine 4 and 5; one between cysteine 3 and 4; and two located at the N-terminus of the peptide. At the N-terminus the sequences reveal a change in a glycine from the HBD3 to a phenylalanine in the Defb14 and a change in isoleucine from the HBD3 to a proline in the Defb14. Between cysteine 3 and 4 the sequence displays a change in proline from the HBD3 to a glycine in the Defb14. In addition a change is observed between cystein 4 and 5 revealing a change from an arginine in the HBD3 to a serine in Defb14.

4.3.2 Expression of Defb14

The expression of *Defb14* was assessed using various tissues from mice. RT-PCR was performed and transcripts were detected in the trachea, oesophagus, and strong expression was evident in the testis and epididymis (Figure 4.1). *Performed by Alison Maxwell.*

PEPTIDE	SEQUENCE	+	ΔG
HBD3	GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK	+11	-12.65
Defb14	FLPKTLRKFFCRIRGGRCAVLNCLGKEEQIGRCSNSGRKCCRKKK	+12	-11.51
Defb14 1cys	FLPKTLRKFFARIRGGRAAVLNALGKEEQIGRASNSGRKCARKKK	+12 (+24)	-13.56

Table 4.1 Sequence, charge and hydrophobicities of HBD3, Defb14 and Defb14 1cys

The above table displays the sequence, charge and hydrophobicity for HBD3, Defb14 and Defb14 1cys
+ indicated net charge of monomer and number in brackets indicates the charge of the dimmer.
Amino acids highlighted in pink denote the changes that are not conserved or semi conserved between Defb14 and HBD3
Those highlighted in blue indicate the conserved cysteine residues and those highlighted in red denote the changes from cysteine to alanine in the dimeric Defb14 1cys. ΔG = hydrophobicity score in water of monomeric peptides calculated using Wimley and White scale, greater hydrophobicity indicated by a less negative value.
Peptides supplied by Albachem Ltd.

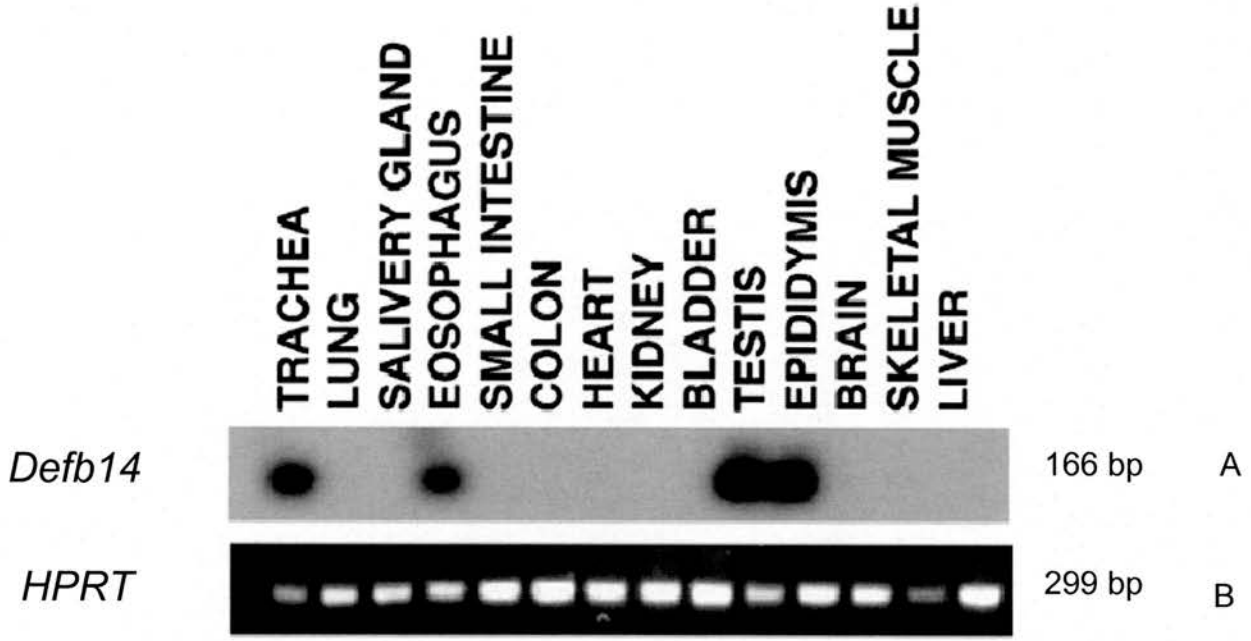


Figure 4.1 Expression of *Defb14* in murine tissues

RNA was isolated from the above tissues and subsequently cDNA was made. cDNA was used to perform PCR reactions for *Defb14* and *HPRT* (positive control) (B). No expression of Defb14 by PCR was detected in the above tissues. As a result southern blot analysis was performed using an internal radiolabelled oligonucleotide for Defb14 (A).
Transcripts were detected in the trachea, oesophagus and strong expression is evident in the testis and epidydimis.

4.3.3 Gel electrophoresis of Defb14

In order to determine the properties of the predicted mature Defb14 peptide encoded by exon 2, we synthesised the 45 amino acid peptide (Table 4.1). Characterisation of the Defb14 peptide on a 16% tricine gel revealed that the preparation consisted of a less predominant upper band with a lower band around 7 kD in size (Figure 4.2). The expected size of Defb14 is 5184 kDa. In contrast, HBD3 has a very different appearance with a lower band running around 5 kDa and additional abundant species at ~ 10 kDa. Upon reduction, the upper band(s) alters in size to ~5 KDa consistent with some significant structural alteration between the reduced form and the oxidised form. To analyse the effect of oligomeric state of Defb14 on its biological properties we synthesised Defb14 1cys wherein all the cysteines were replaced with alanine residues except from that at position 5 (Table 4.1). The majority (>95%) of Defb14 1cys oxidised peptide migrates with an apparent mass of ~ 12 kDa (with a small amount of peptide at ~5 kDa) and upon reduction, the major band appeared at 7 kDa.

4.3.4 Accurate mass analysis

Defb14 and Defb14 1cys were analysed by ESI-MS to assess their aggregation tendencies and high resolution ESI-FT-ICR mass spectrometry was used to determine the oxidation states of Defb14 and HBD3 (Performed and optimised by Bryan McCullough). Figure 4.3 shows the ESI mass spectra of Defb14 (top) and of Defb14 1cys (bottom) obtained from analysis on a Q-ToF MS (Micromass). Figure 4.4 shows the isotopic envelopes obtained for the +6 charge states of Defb14 (top) and HBD3 (bottom) following FT-ICR MS analysis. The open squares are the predicted isotopic ditributions from the sequence of a fully oxidised peptide with three S-S bridges and the closed squares are the predicted distribution of isotopes for a fully-reduced peptide. It is clear that there is very good agreement between the theoretical and observed distribution showing that both peptides are fully oxidised.

Defb14 is monomeric whereby Defb14 1 cys readily oxidised to form a dimer with a single intramolecular disulfide bond shown by mass spectrometry (Figure 4.3 bottom). Defb14 1cys also presents an isotopic distribution by FT-ICR MS which

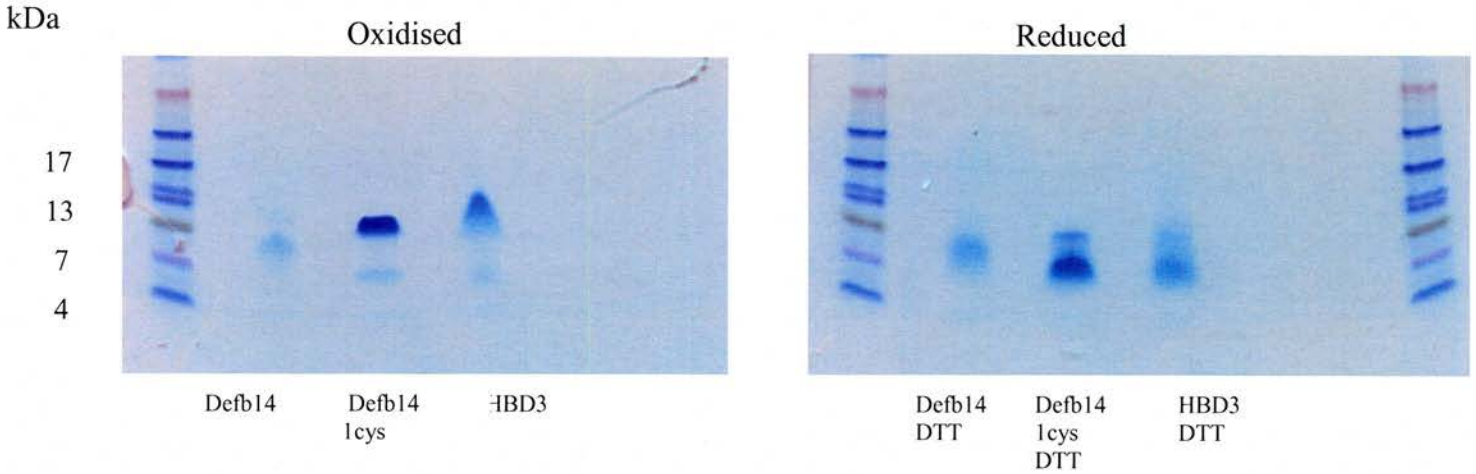


Figure 4.2 Gel electrophoresis of Defb14, Defb14 1cys and HBD3

The above 16% tricine gels display Defb14, Defb14 1cys and HBD3 in the absence (oxidised) and presence (reduced) of 10 mM DTT.

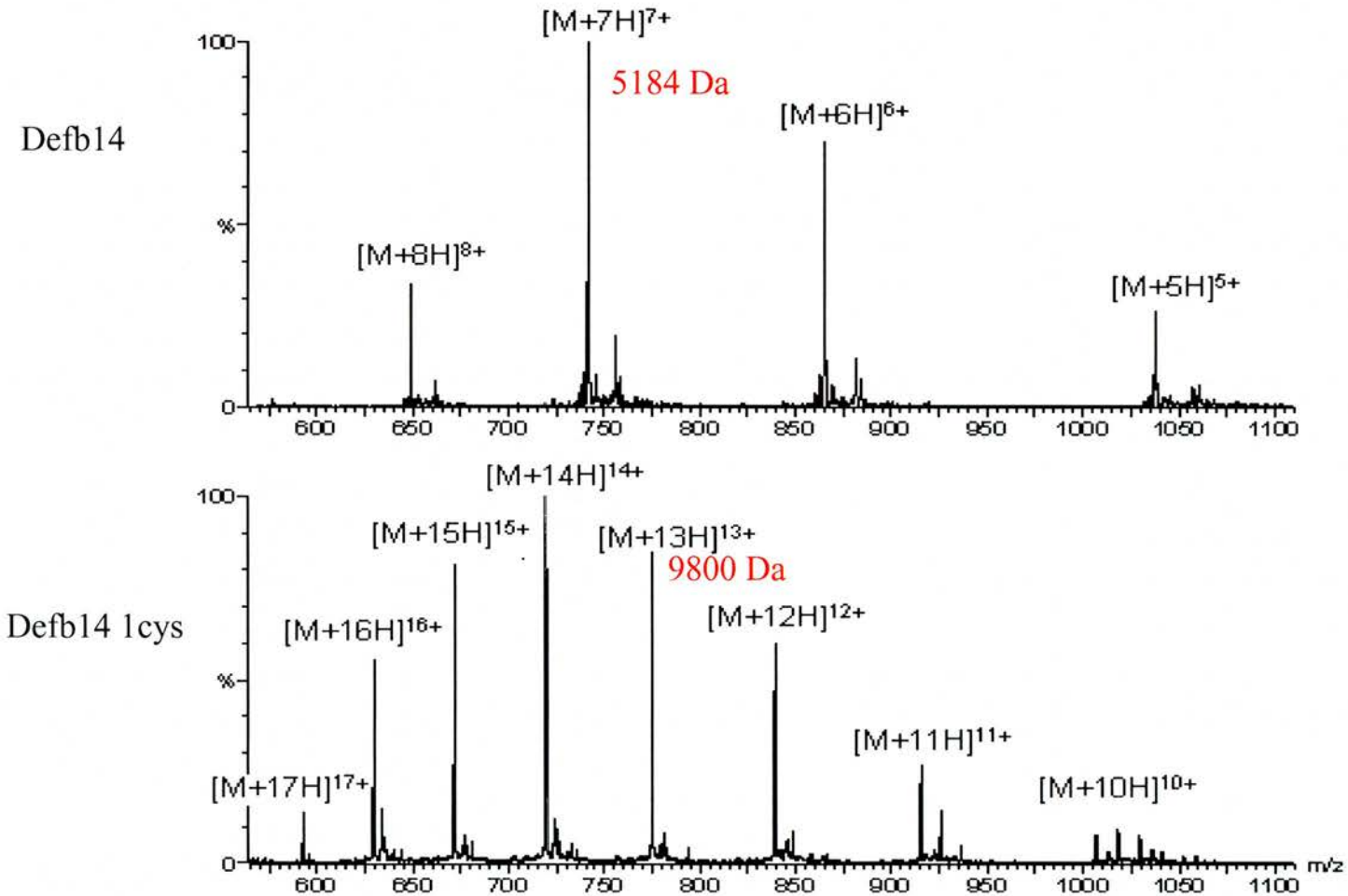
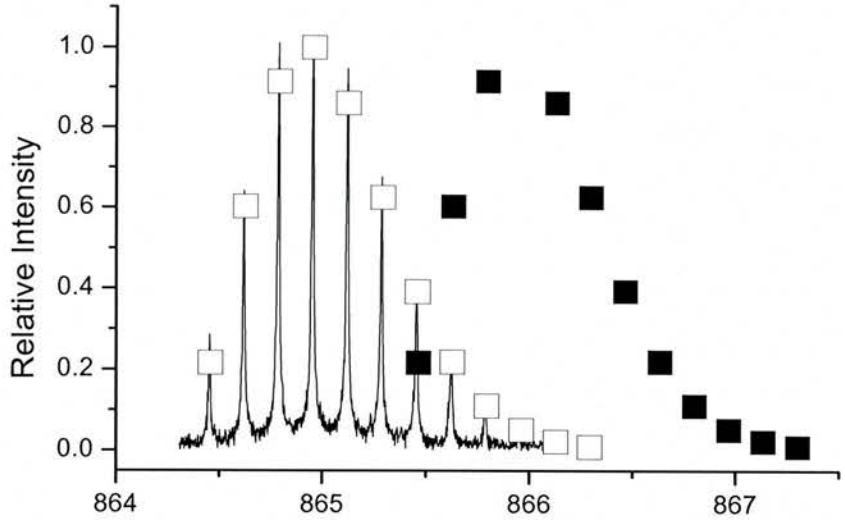


Figure 4.3 Deconvoluted mass spectrometry of Defb14 (top) and Defb14 1cys (bottom)

ESI-FT-ICR analysis on a Q-ToF. Predicted mass for Defb14 $[M+6H]^{6+}$ =5114, actual mass = 5184 Da. Predicted mass for Defb14 1cys $[M+13H]^{13+}$ = 10000, actual mass = 9800 Da. For Defb14 isotopic distribution refer to Figure 4.4.

Defb14
[M+6H] $^{6+}$



HBD3

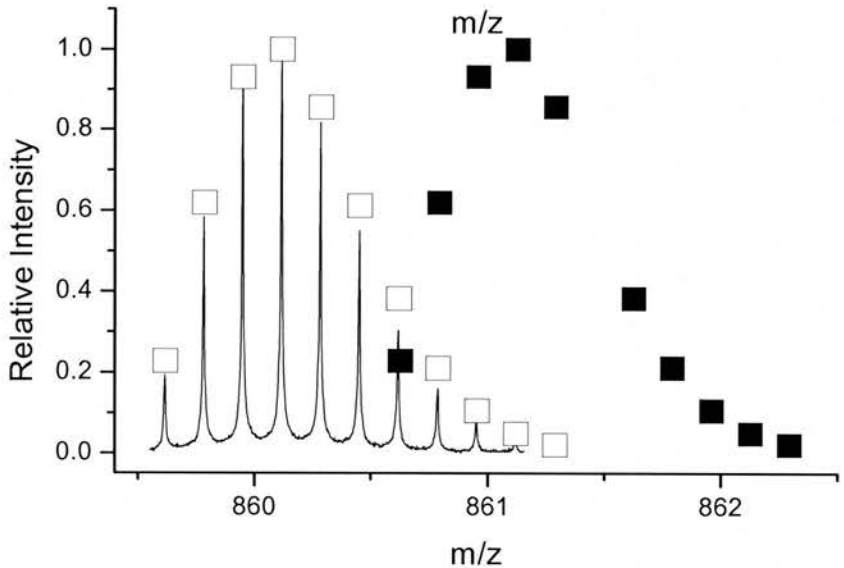


Figure 4.4 Isotopic distribution following FT-ICR MS analysis

This figure shows the m/z of H. The open squares are the predicted distribution of isotopes for a oxidised peptide. The closed squares are the predicted distribution of isotopes for a reduced peptide. For Defb14 refer to Figure 4.3 [M=6H] $^{6+}$ for deconvoluted mass spectrometry.

corresponds to a fully oxidised peptide, which strongly implies that this is a disulfide bridge dimer (data not shown). This was confirmed by CID (collsion-induced dissociation) MS analysis (Perdita Barran personal communication); the peptide does not dissociate into two monomers under these conditions suggesting a covalent interaction. This is further confirmed by gel electrophoresis analysis that also reveals structural alteration between the reduced and oxidised forms of this peptide.

4.3.5 Disulfide analysis

Following digestion and mass mapping, the S-S connectivities observed between cysteines in Defb14 and in HBD3 are shown in table 4.2 (a-c) (Performed and optimised by Dr David Clarke). Table 4.2a represents peptides from a Defb14 trypsin digest. Sequences in brackets indicate the presence of S-S bonds either within the peptide (one sequence) or between peptides (two sequences). Two major connectivities were assigned Cys1-Cys4, Cys2-Cys3, Cys5-Cys6 and either Cys1-Cys4, Cys2- Cys5, Cys3-Cys6 or Cys1-Cys4, Cys2-Cys6, Cys3-Cys5 (these were indistinguishable by mass alone). Table 4.2b displays the peptides identified when one missed cleavage is taken into account; this only shows evidence for the Cys1-Cys4, Cys2-Cys3, Cys5-Cys6 topology suggesting it is the most abundant connectivity. Table 4.2c displays the identified fragments from a trypsin/chymotrypsin digestion of HBD3. Only the N-terminus and the canonical Cys2-Cys4 peptide were able to be assigned.

4.3.6 NMR analysis

A comparison of the 1H 600 MHz spectra of Defb14 and HBD3 (Figure 4.5) was performed and optimised by Dr Dusan Uhrin. Figure 4.5 shows large differences in the spread of the 1H signals between the two peptides. Signals at both extremes of the HBD3 spectrum are in agreement with the reported 1H chemical shifts for canonically folded HBD3 (Schibli *et al.*, 2002). With Defb14 a much narrower range of NH, aromatic and CH3 signals found for Defb14 indicated that this fully oxidised peptide with 3 S-S bonds, does not impose a unique and stable tertiary structure. Defb14 was found to exist as a random coil whereas HBD3 possesses chemical shifts which contain

a

FRAGMENT NUMBER	SEQUENCE	EXPECTED MASS	MEASURED MASS
1	FLPK	503.31	503.32
2	TLR	388.24	388.34
3	K	146.11	146.08
4+9	(FFC1R)(C4SNSGR)	1191.5	1191.45
5	IR	287.2	287.21
6	GGR	288.15	288.18
7	(C2AVLNC3LGK)	917.45	917.47
7+11	(C2AVLNC3LGK)(C5C6K)	1295.57	1295.86
8	EEQIGR	730.36	730.29
10	K	146.11	146.08
11	(C5C6K)	378.12	378.52
12	K	146.11	146.08
13	K	146.11	146.08
14	K	146.11	146.08

b

FRAGMENT NUMBER	SEQUENCE	EXPECTED MASS	MEASURED MASS
1+2	FLPKTLR	873.55	873.65
2+3	TLRK	516.35	516.97
3+4+9	(KFFC1R)(C4SNSGR)	1319.61	1319.96
4+5+9	(FFC1RIR)(C4SNSGR)	1461.69	1461.33
7+8	(C2AVLNC3LGKEEQIGR)	1630.91	1630.78

c

FRAGMENT NUMBER	SEQUENCE	EXPECTED MASS	MEASURED MASS
1	GIINTL	629.37	629.42
8+11	(C2AVL)(C4STR)	388.24	388.34

Table 4.2 (a-c) Identified tryptic fragments of Defb14 and HBD3

Table 4.2a displays the identified peptides from Defb14 trypsin digest.
Table 4.2b displays the peptides identified when one missed cleavage is taken into account.
Table 4.2c displays the identified fragments from a trypsin/chymotrypsin digestion of HBD3.

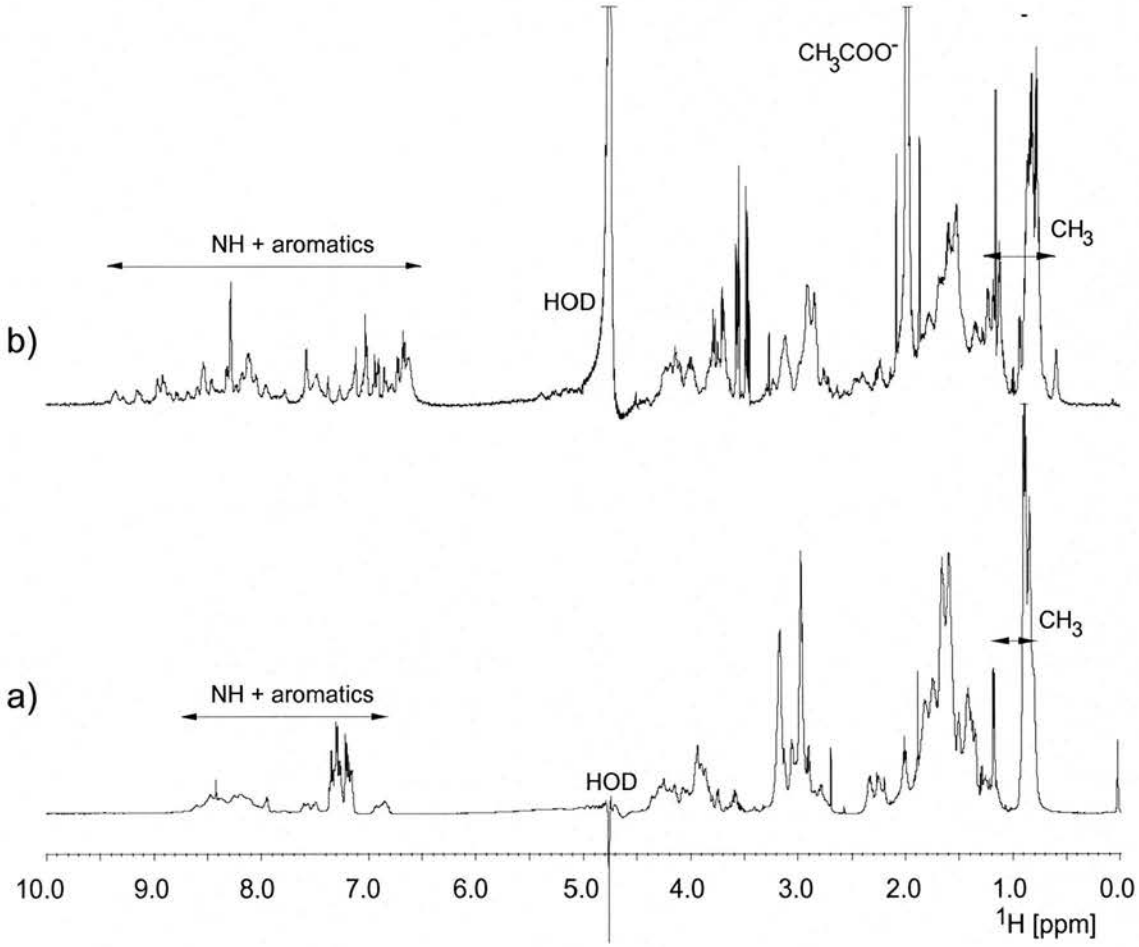


Figure 4.5 NMR analysis of Defb14 and HBD3

The above figure displays the NMR analysis of HBD3 (b) and Defb14 (a). Signals at both extremes of the spectrum are in agreement with the reported shifts for canonically folded HBD3 (b). With Defb14 (a) a narrower range of NH and CH3 signals indicate that this is a fully oxidised peptide with 3 S-S bonds exisiting as a random coil indicating no tertiary stable structure.

the characteristic three stranded β-sheet core with a partly helical segment at the N-terminus. Accurate mass analysis was performed before and after the NMR to rule out protein reduction or degradation during the experiment.

4.3.7 Antimicrobial analysis of Defb14 and Defb14 1cys

Antimicrobial analysis was performed on both Defb14 and Defb14 1cys against a panel of Gram positive and negative micro-organisms. Defb14 displays potent antimicrobial activity against a spectrum of organisms (Figure 4.6 - 4.12). The majority of organisms gave MBC values with Defb14 (Table 4.3). *P.aeruginosa* PAO1 appeared to be the most susceptible with an MBC value of 1.5 μg/ml ($p<0.01$) and *B.cenocepacia* the most resistant with an MBC value greater than 100 μg/ml. Furthermore, the activity of the single cysteine dimer form of the peptide (Defb14 1cys) displays similar MBC values as the original Defb14, *P.aeruginosa* PAO1 1.5 μg/ml ($p<0.01$) and yet again high values against *B.cenocepacia* >100 μg/ml. Upon reduction with DTT no change was evident in the MBC values from both the Defb14 and Defb14 1cys (Table 4.3).

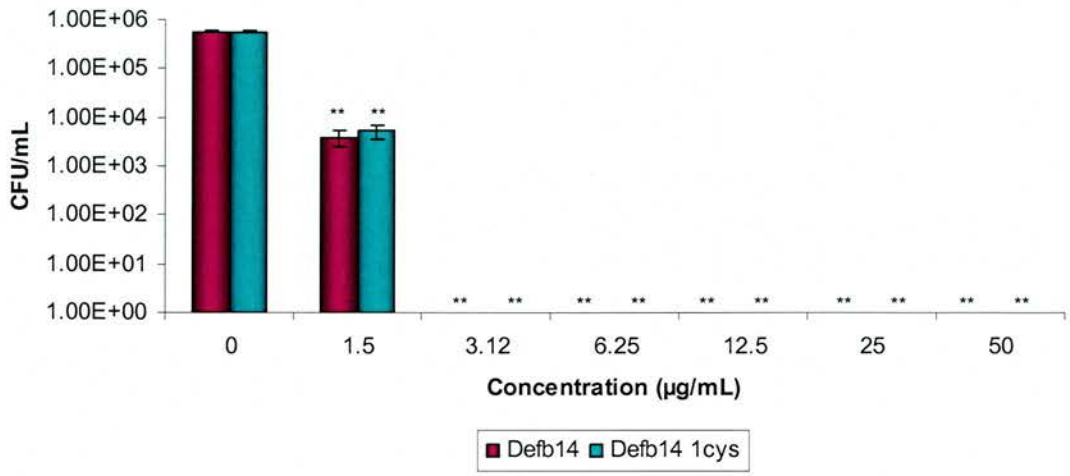
4.3.8 Evaluation of varying salt concentrations on antimicrobial activity

To identify the effect of NaCl on the antimicrobial activity of Defb14 and Defb14 1cys varying salt concentrations were evaluated (Table 4.4). Defb14 and Defb14 1cys were tested with *S.aureus* and *P.aeruginosa* against 50 mM to 250 mM concentrations of NaCl. Both peptides displayed antimicrobial activity with *S.aureus* but higher concentrations were required for killing at 250 mM. MBC values increase from 3.13 μg/ml to 25 μg/ml at this salt concentration. In contrast the MBC values for *P.aeruginosa* remained unaltered at 1.5 μg/ml for the full range of salt concentrations.

4.3.9 Evaluation of varying human serum concentrations on antimicrobial activity

To identify the effect of human serum on the antimicrobial values of Defb14 and Defb14 1cys varying concentration of serum were evaluated. Pooled human serum was

A



B

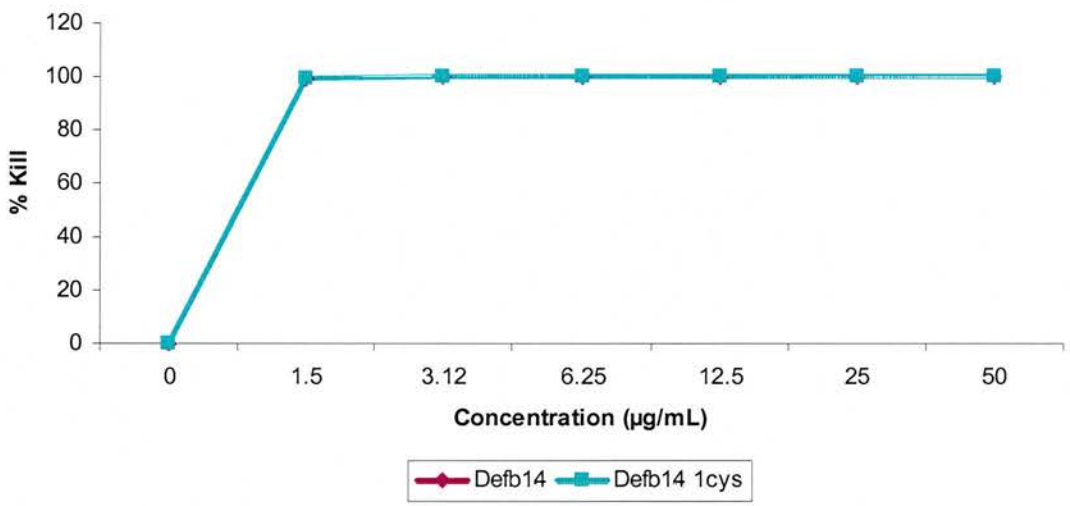
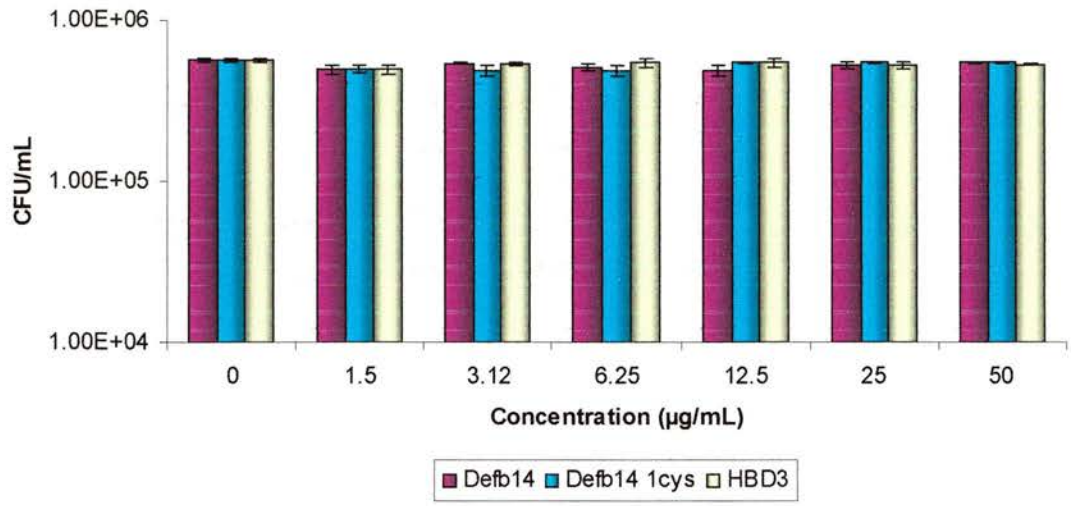


Figure 4.6 Antimicrobial activity of Defb14 and Defb14 1cys against *A. baumannii* ATCC 19606

Graph A shows the number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

A



B

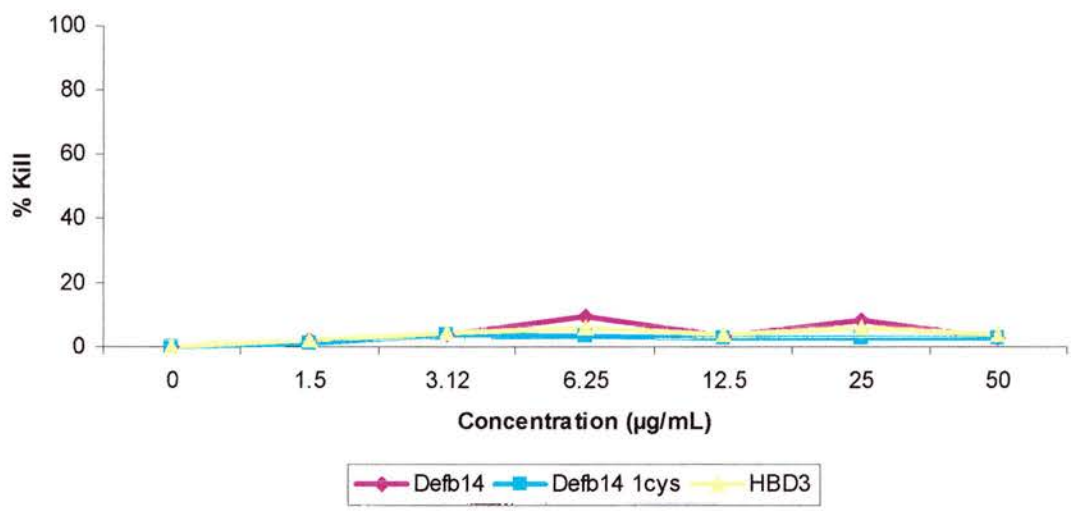
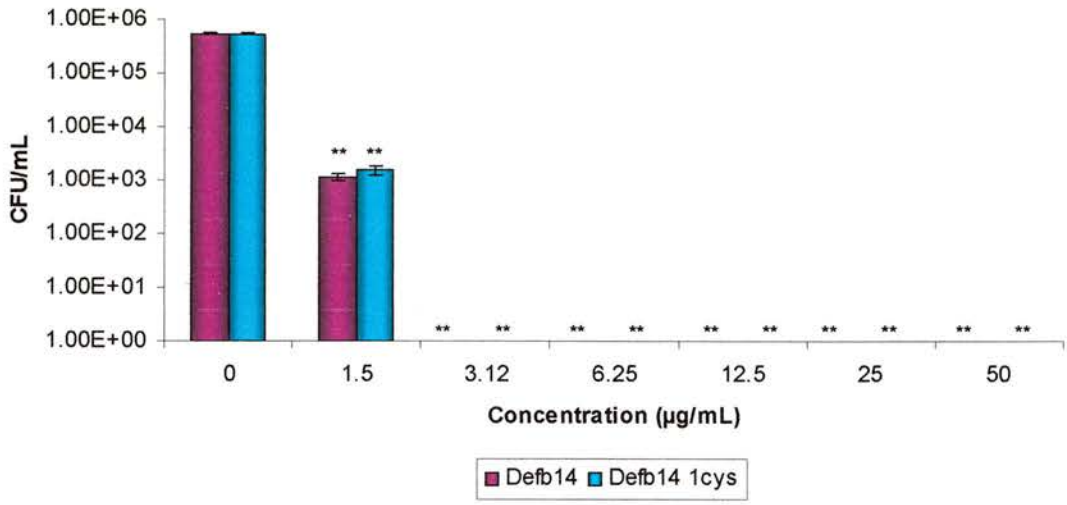


Figure 4.7 Antimicrobial activity of Defb14, Defb14 1cys and HBD3 against *B. cenocepacia* J2315

Graph A shows the number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were performed in triplicate and on three independent occasions (n=9).

A



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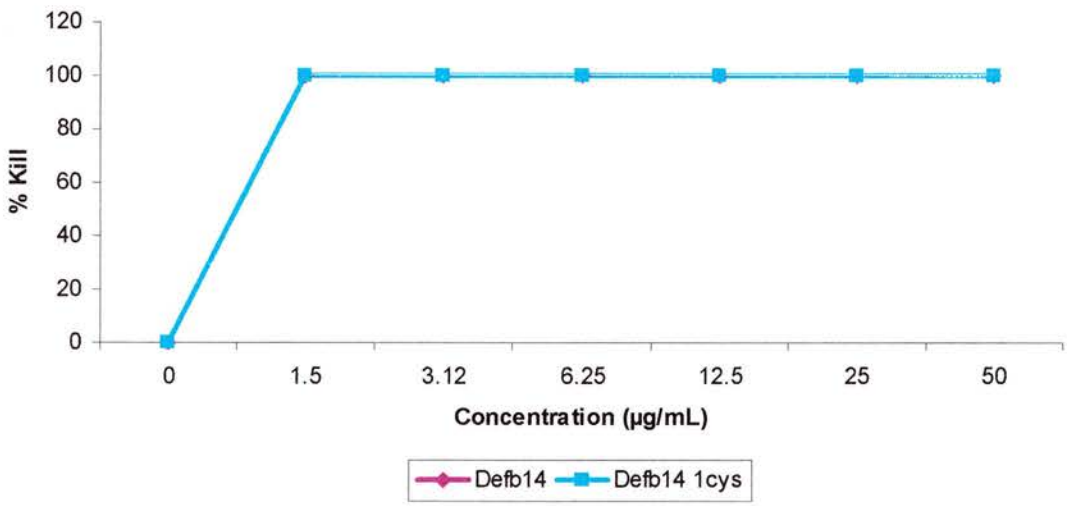
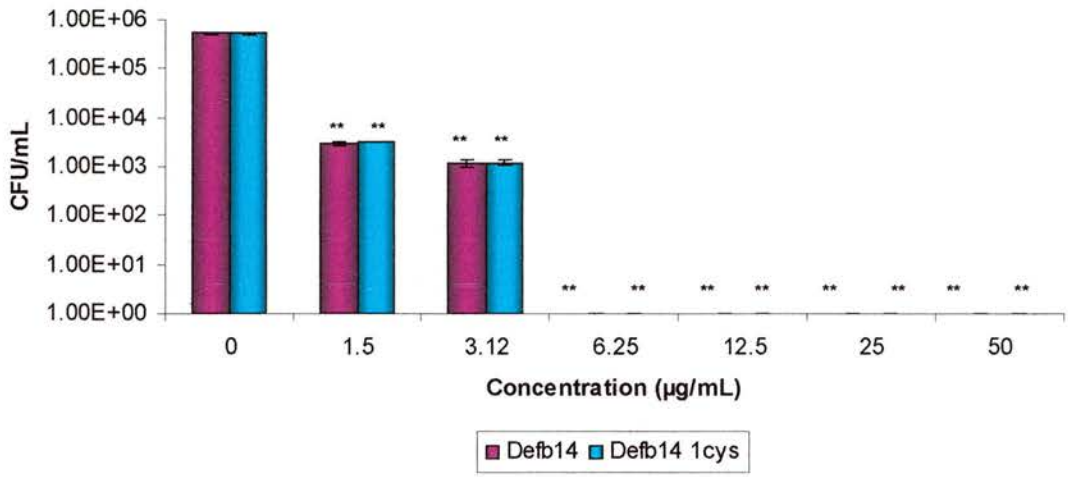


Figure 4.8 Antimicrobial activity of Defb14 and Defb14 1cys against *E. coli* ATCC 25922

Graph A shows the number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were performed in triplicate and on three independent occasions (n=9).

A



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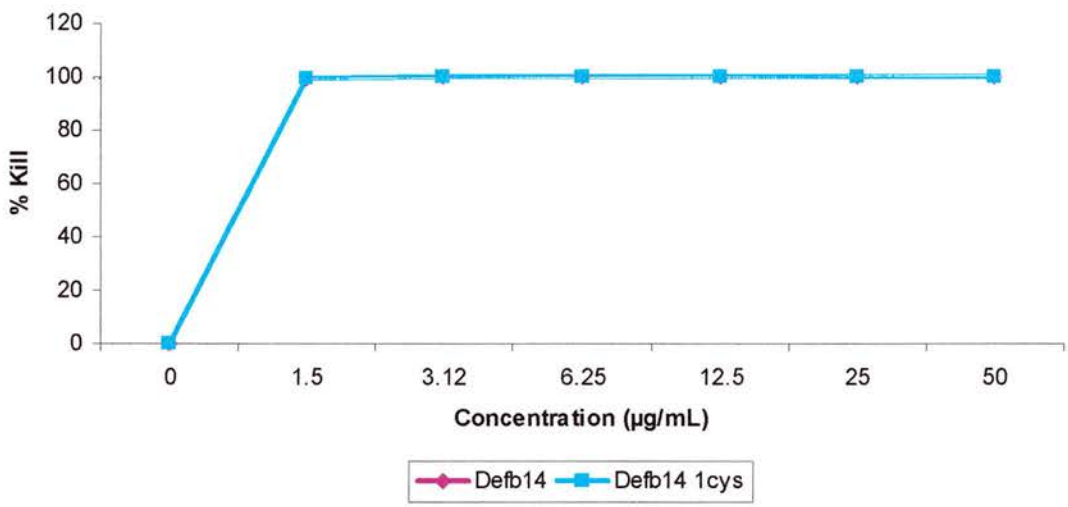


Figure 4.9 Antimicrobial activity of Defb14 and Defb14 1cys against *E. faecalis* ATCC 700802

Graph A shows the number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were performed in triplicate and on three independent occasions (n=9).

A

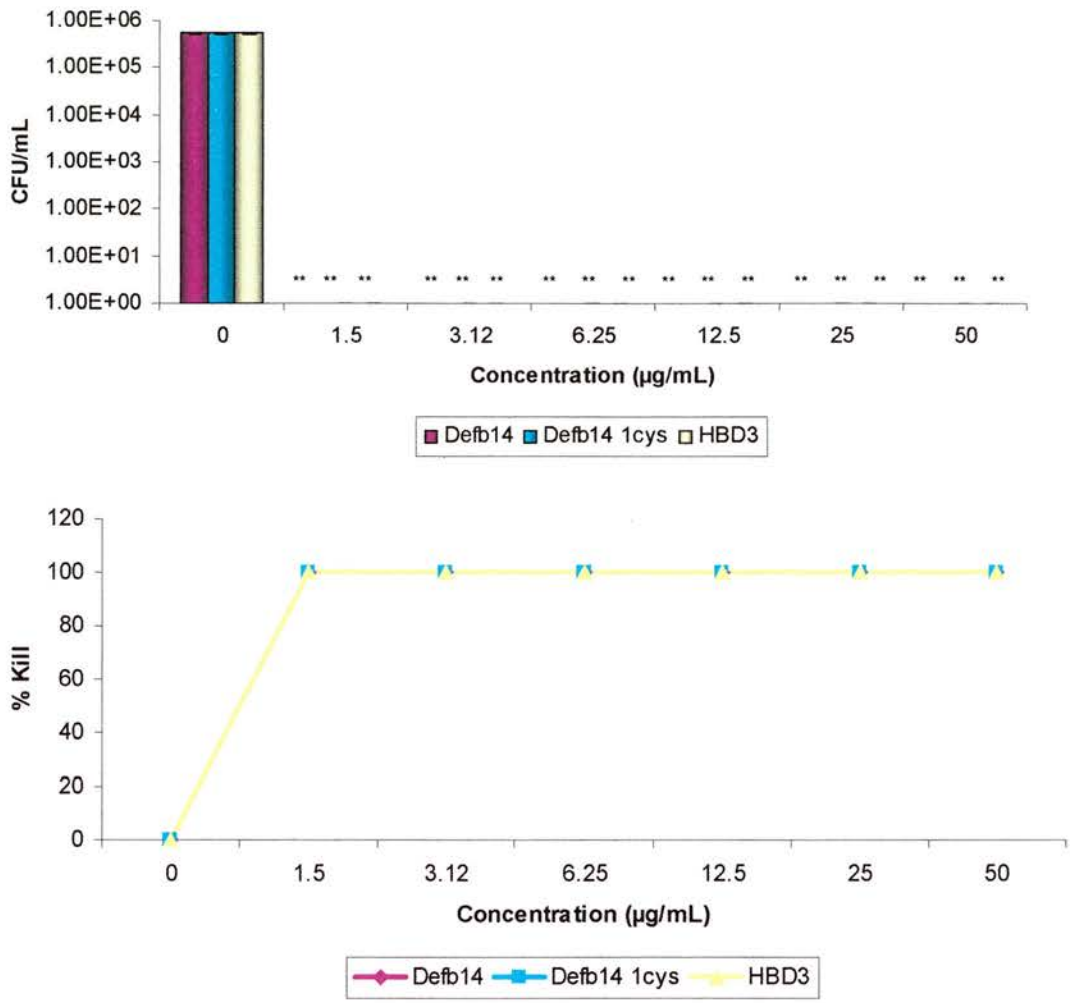
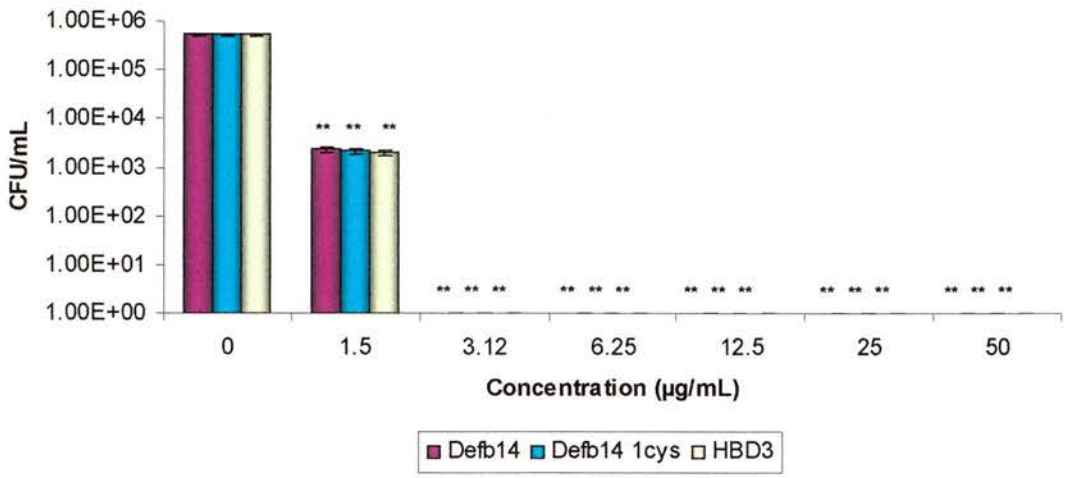


Figure 4.10 Antimicrobial activity of Defb14, Defb14 1cys and HBD3 against *P. aeruginosa* PAO1

Graph A shows the number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were performed in triplicate and on three independent occasions (n=9).

A



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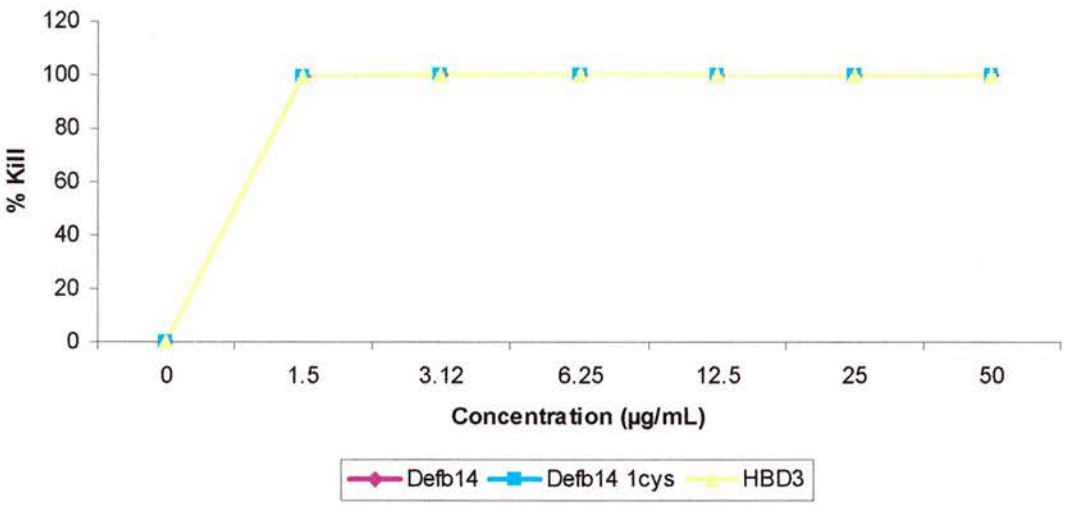
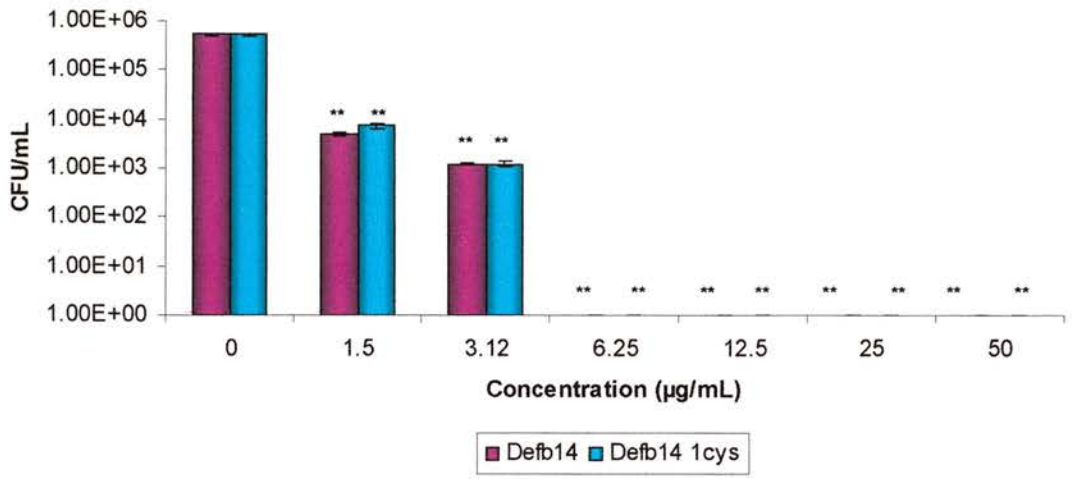


Figure 4.11 Antimicrobial activity of Defb14, Defb14 1cys and HBD3 against *S. aureus* ATCC 25923

Graph A shows the number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were performed in triplicate and on three independent occasions (n=9).

A



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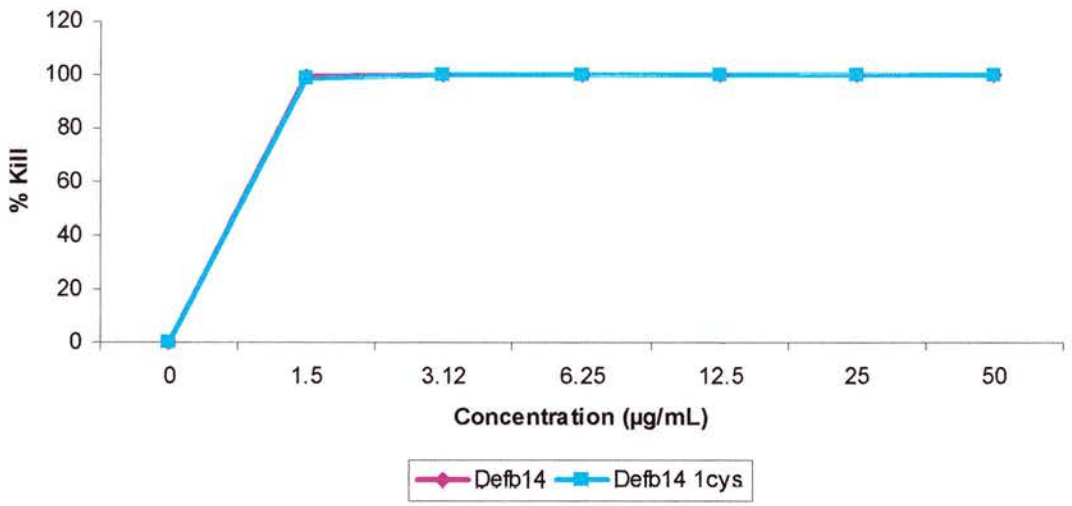


Figure 4.12 Antimicrobial activity of Defb14 and Defb14 1cys against *S. aureus* MRSA J2918

Graph A shows the number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were performed in triplicate and on three independent occasions (n=9).

ORGANISM	Defb14	DTT	Defb14 1cys	DTT	HBD3	DTT
A .baumannii ATCC 19606	3.13	-	3.13	-	-	-
B. cenocepacia J2315	>50	>50	>50	>50	>50	>50
E .coli ATCC25922	3.13	-	3.13	-	-	-
E. faecalis ATCC 700802	6.25	-	6.25	-	-	-
P. aeruginosa PAO1	1.5	1.5	1.5	1.5	1.5	1.5
S. aureus ATCC 25923	3.13	3.13	3.13	3.13	3.13	3.12
S. aureus MRSA J2918	6.25	-	6.25	-	-	-

Table 4.3 Minimum bactericidal values of Defb14, Defb14 1cys and HBD3

The antimicrobial activity of Defb14, Defb14 1cys and HBD3 was evaluated against a panel of microorganisms. Peptides were treated with and without 10mM DTT prior to testing. Minimum Bactericidal Concentrations presented as µg/ml. Not tested represented as – . Values in red treated with 10mM DTT

NaCl (mM)	*S.aureus* ATCC 25923		*P.aeruginosa* PAO1	
	Defb14	**Defb14 1cys**	**Defb14**	**Defb14 1cys**
0	3.13	3.13	1.5	1.5
50	3.13	3.13	1.5	1.5
100	6.25	6.25	1.5	1.5
150	12.5	12.5	1.5	1.5
200	12.5	12.5	1.5	1.5
250	25	25	1.5	1.5

Table 4.4 Minimum bactericidal values of Defb14 and Defb14 1cys in various salt conditions

The antimicrobial activity of Defb14 and Defb14 1cys were evaluated in the presence of varying concentrations of NaCl. Both peptides were tested against *P. aeruginosa* PAO1 and *S. aureus* ATCC 25923 and minimum bactericidal concentrations represented as μg/ml.

used at concentrations of 0.1 %, 1 % and 10 %. Defb14 and Defb14 1 cys were evaluated against *P. aeruginosa* PAO1 at a peptide concentration of 50 μg/mL. In the presence of 0.1 %, 1 % and 10 % serum the antimicrobial activity was significantly potent (0.1 % ($p<0.01$), 1% ($p<0.01$) and 10 % ($p<0.01$) comparable with the 0 % serum free control.

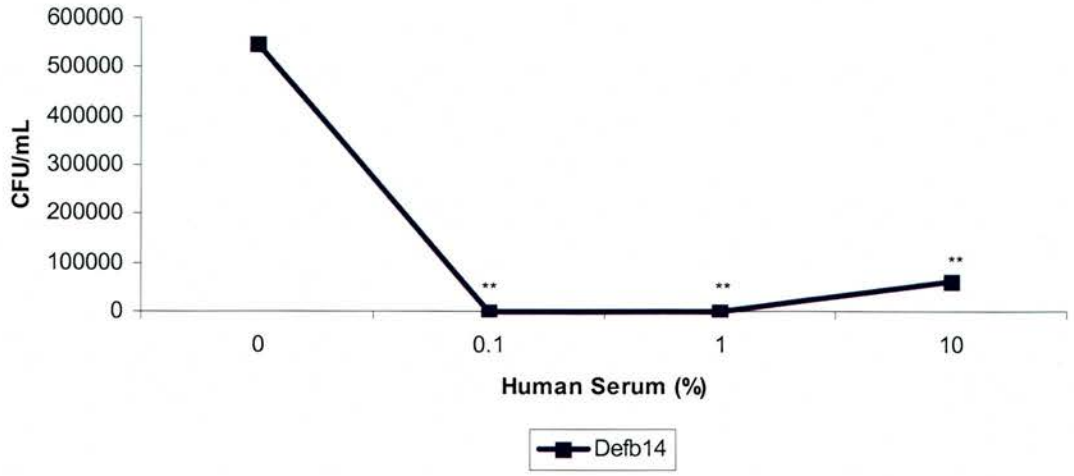
4.3.10 Chemoattractant analysis of Defb14 and Defb14 1cys

Both Defb14 and Defb14 1cys were evaluated for chemotactic activity against neutrophils, monocytes, CD4+ T cells (human & mouse) and CCR6 expressing HEK293 cells (Figure 4.14-4.19). The Defb14 parent monomeric molecule displays activity on human monocytes (Table 4.5) at 1000 ng/ml ($p<0.01$) with the Defb14 1cys displaying optimum activity at 100 ng/ml ($p<0.01$). Defb14 and Defb14 1cys are also able to chemoattract human and mouse CD4 T cells at 100 ng/ml ($p<0.01$) and 10 ng/ml ($p<0.01$) respectively. Again the single cysteine dimer displays ten fold greater activity. Figure 4.17 show that Defb14, similar to HBD3 induces the chemotaxis of CCR6-expressing HEK293 cells in a dose dependent manner with an optimal concentration of 100 ng/ml ($p<0.01$). Defb14 and Defb14 1cys are equally active against the human CCR6 receptor on the HEK293 cells. No migration was observed from both the Defb14 and Defb14 1cys against murine neutrophils (Figure 4.19).

4.3.11 Haemolytic activity of Defb14 and Defb14 1cys

The haemolytic activity of Defb14 and Defb14 1cys were evaluated against human erythrocytes (Figure 4.20). A range of concentrations from 3.9 μg/ml to 500 μg/ml were tested with both Defb14 and Defb14 1cys giving similar values observed haemolysis taking place at concentrations above 250 μg/ml. In addition peptides Defb14 and Defb14 1cys treated with DTT were evaluated. Both the peptides gave similar values to each other and to that of the peptide not treated with DTT.

A



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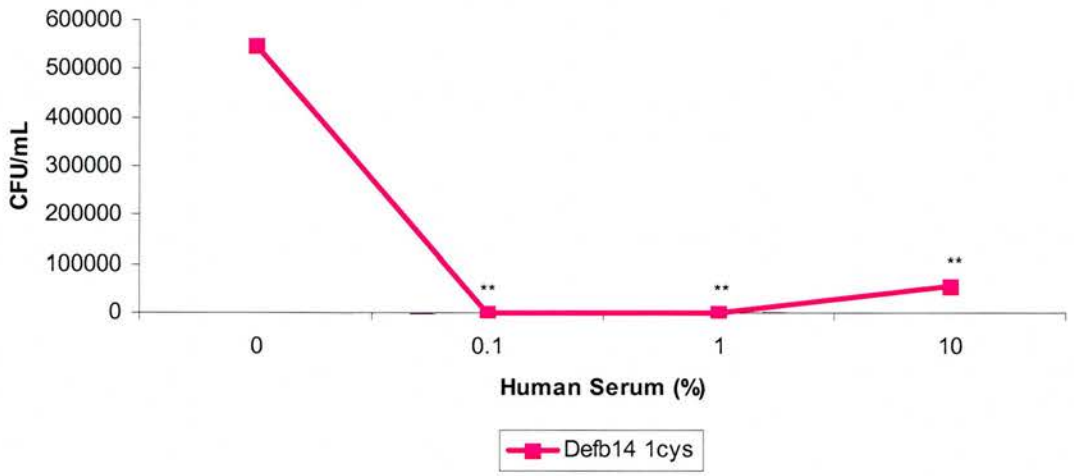
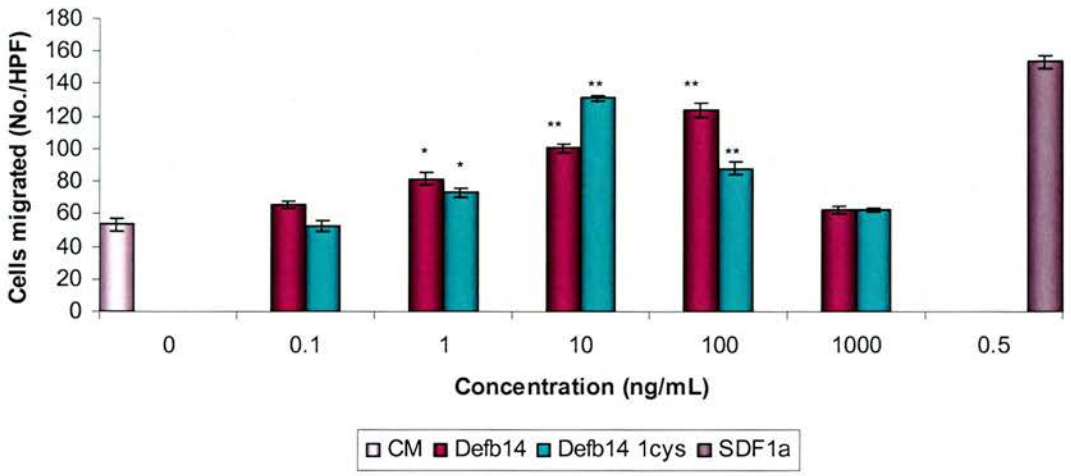


Figure 4.13 Antimicrobial activities of Defb14 against *P. aeruginosa* PAO1 in the presence of human serum

Bacteria were incubated with the peptide at 50μg/mL over a range of human serum percentages. Graph A and B shows the number of colony forming units (CFU) ± standard error from the blank control and peptide treated samples. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

A



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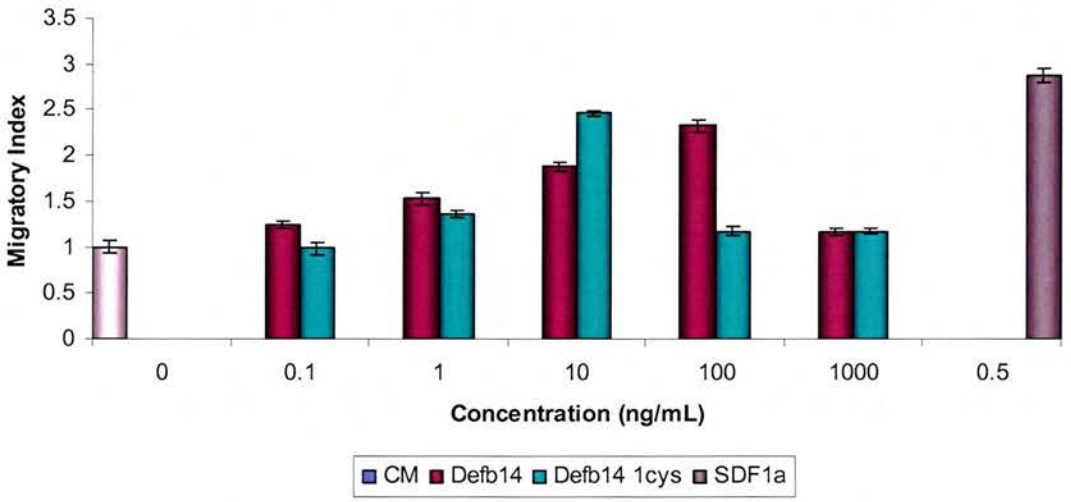
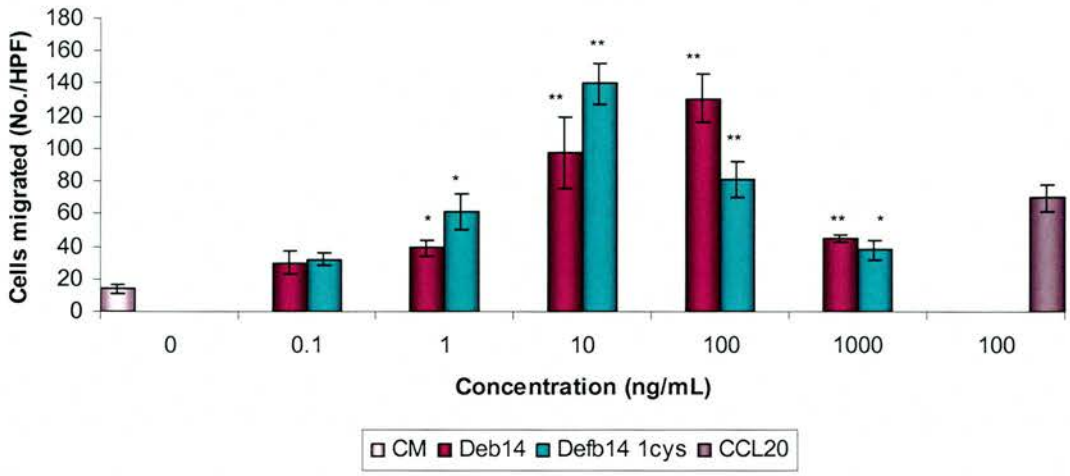


Figure 4.14 Migration of mouse CD4+ T cells in response to Defb14 and Defb14 1cys

Graph A shows the mean number of cells migrated per random field of view. Graph B shown the migratory index, the number of cells migrated in each sample divided by the number of migrated cells in the media alone sample. * represents $p < 0.05$, ** $p < 0.01$. Error bars represent the standard error of all nine replicate counts. Experiments were repeated three times and the above is a representative experiment (n=3).

A



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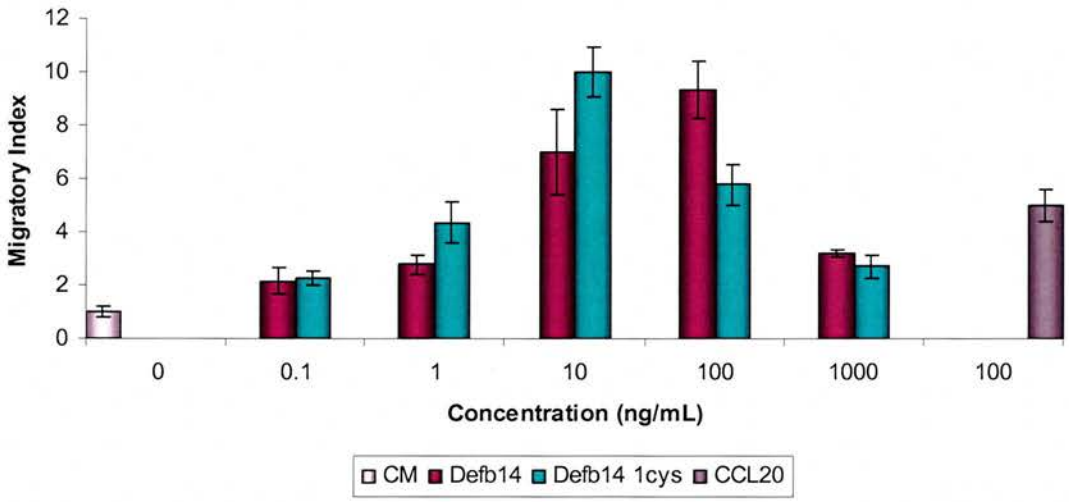
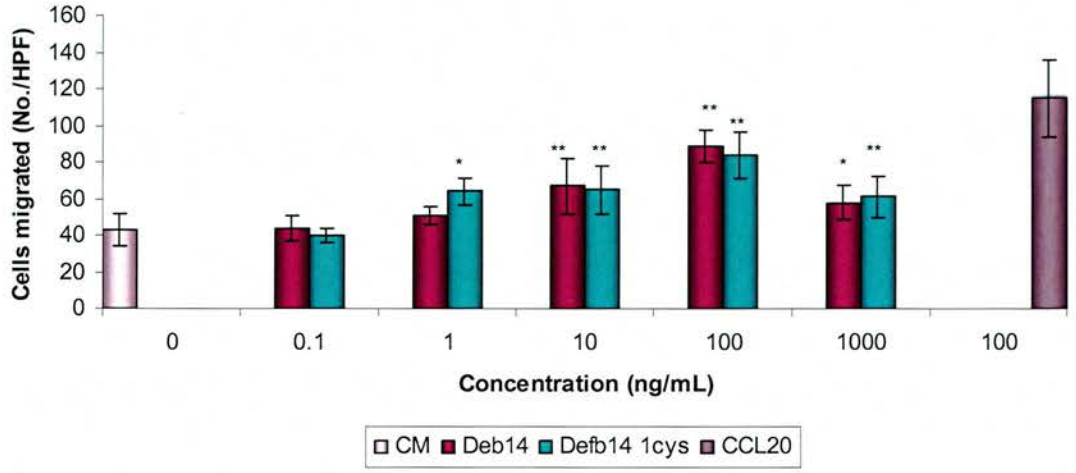


Figure 4.15 Migration of human CD4+ T cells in response to Defb14 and Defb14 1cys

Graph A shows the mean number of cells migrated per random field of view. Graph B shown the migratory index, the number of cells migrated in each sample divided by the number of migrated cells in the media alone sample. * represents $p< 0.05$, ** $p<0.01$. Error bars represent the standard error of all nine replicate counts. Experiments were repeated three times and the above is a representative experiment (n=3).
Chemotaxis peformed by De Yang, Frederick USA.

A



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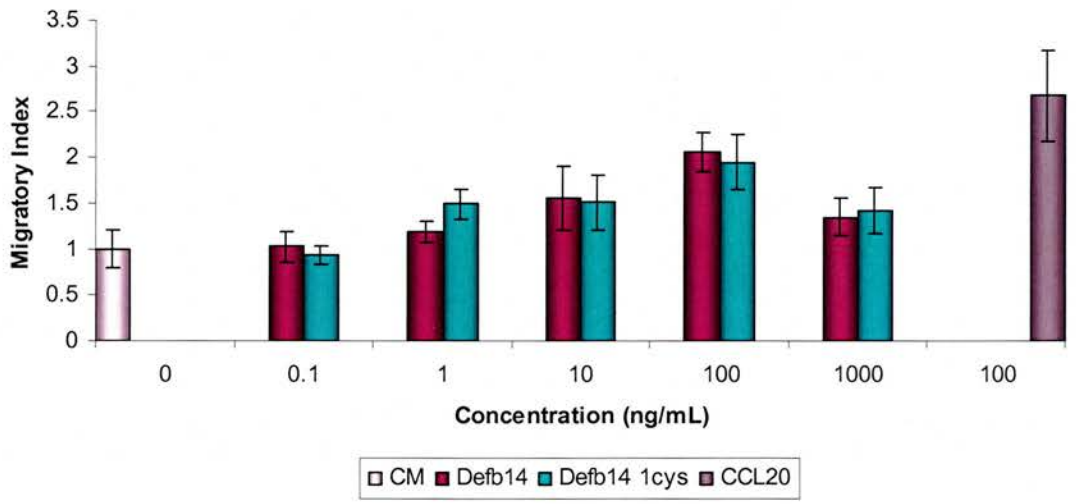
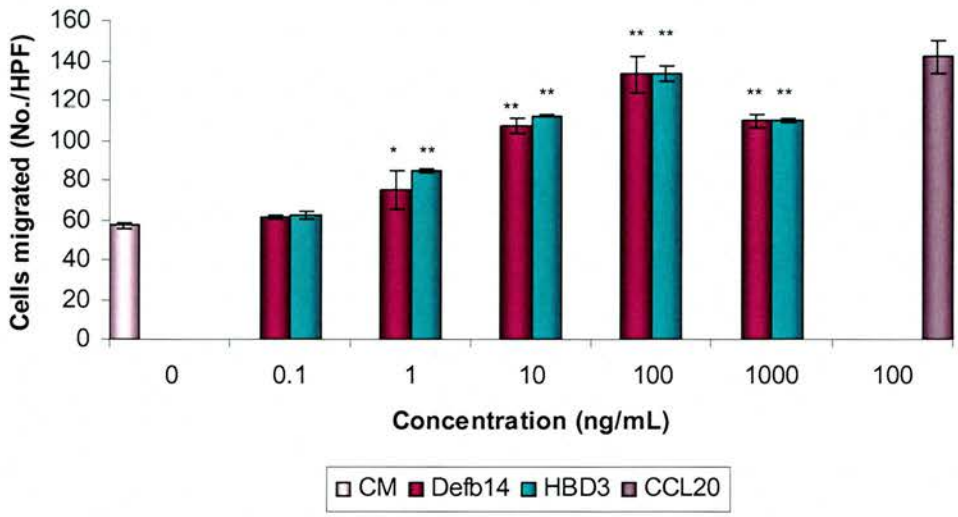


Figure 4.16 Migration of CCR6 expressing HEK293 cells in response to Defb14 and Defb14 1cys

Graph A shows the mean number of cells migrated per random field of view. Graph B shown the migratory index, the number of cells migrated in each sample divided by the number of migrated cells in the media alone sample. * represents $p< 0.05$, ** $p<0.01$. Error bars represent the standard error of all nine replicate counts Experiments were repeated three times and the above is a representative experiment (n=3).

A



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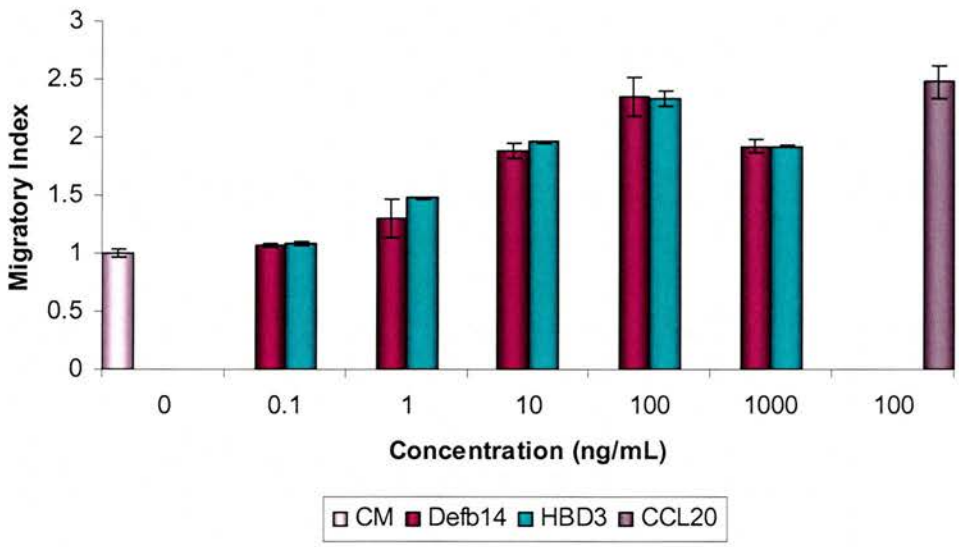
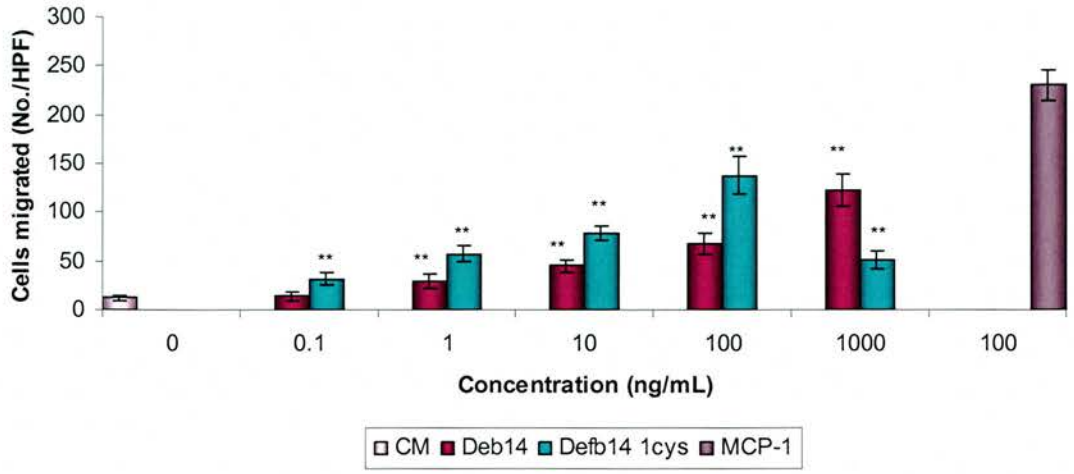


Figure 4.17 Migration of CCR6 expressing HEK293 cells in response to Defb14 and HBD3

Graph A shows the mean number of cells migrated per random field of view. Graph B shown the migratory index, the number of cells migrated in each sample divided by the number of migrated cells in the media alone sample. * represents $p < 0.05$, ** $p < 0.01$. Error bars represent the standard error of all nine replicate counts. Experiments were repeated three times and the above is a representative experiment (n=3).

A



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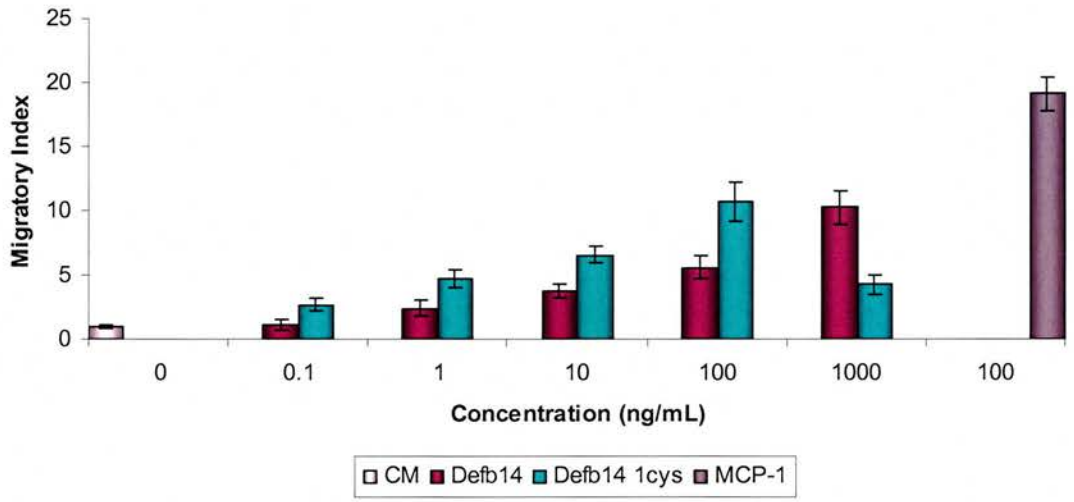
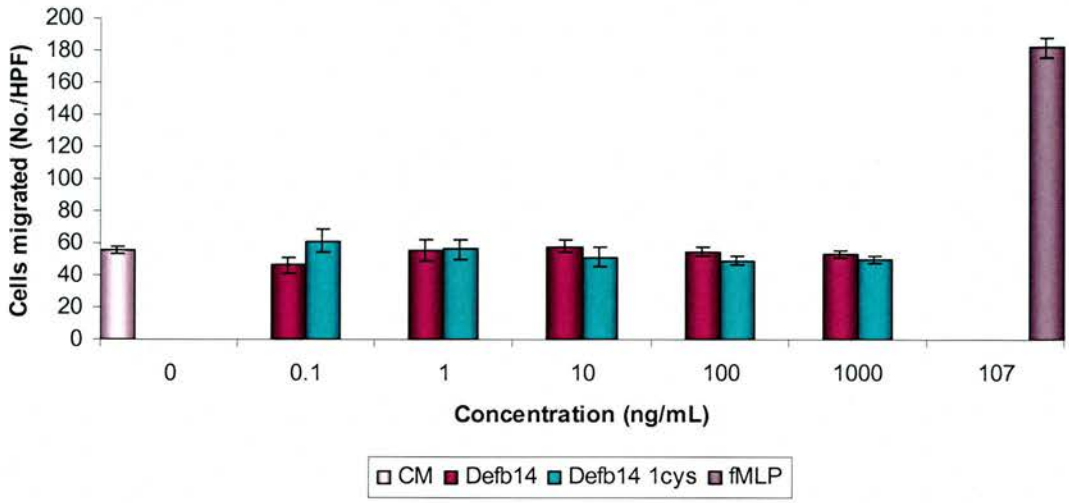


Figure 4.18 Migration of human monocytes in response to Defb14 and Defb14 1cys

Graph A shows the mean number of cells migrated per random field of view. Graph B shown the migratory index, the number of cells migrated in each sample divided by the number of migrated cells in the media alone sample. * represents $p< 0.05$, ** $p<0.01$. Error bars represent the standard error of all nine replicate counts. Experiments were repeated three times and the above is a representative experiment (n=3).
Chemotaxis performed by De Yang, Frederick USA.

A



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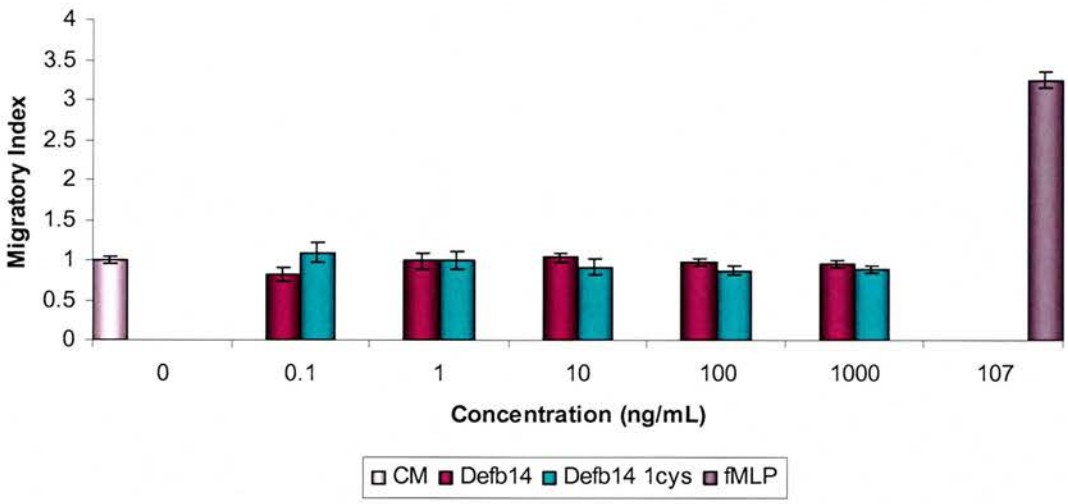
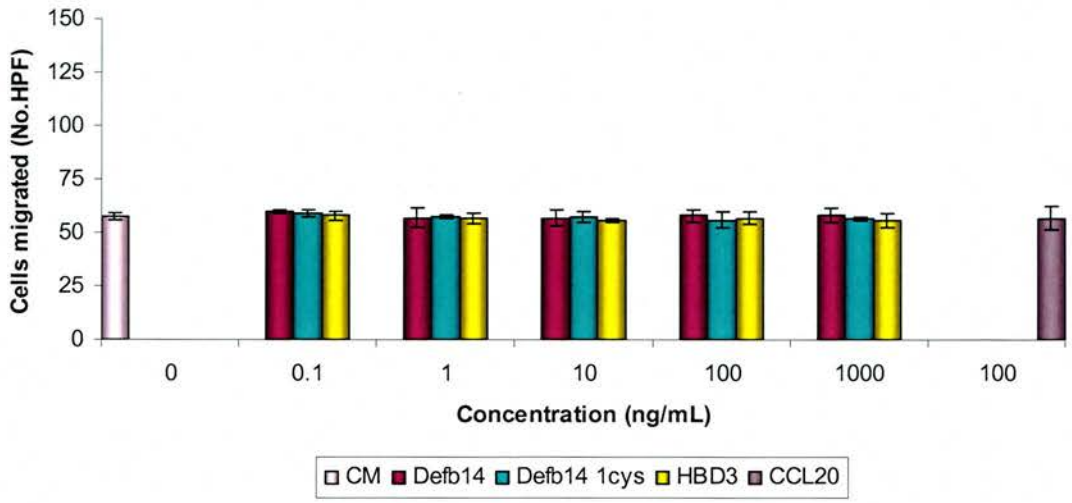


Figure 4.19 Migration of neutrophils in response to Defb14 and Defb14 1cys

Graph A shows the mean number of cells migrated per random field of view. Graph B shown the migratory index, the number of cells migrated in each sample divided by the number of migrated cells in the media alone sample. * represents $p< 0.05$, ** $p<0.01$. Error bars represent the standard error of all nine replicate counts. Experiments were repeated three times and the above is a representative experiment (n=3).

A



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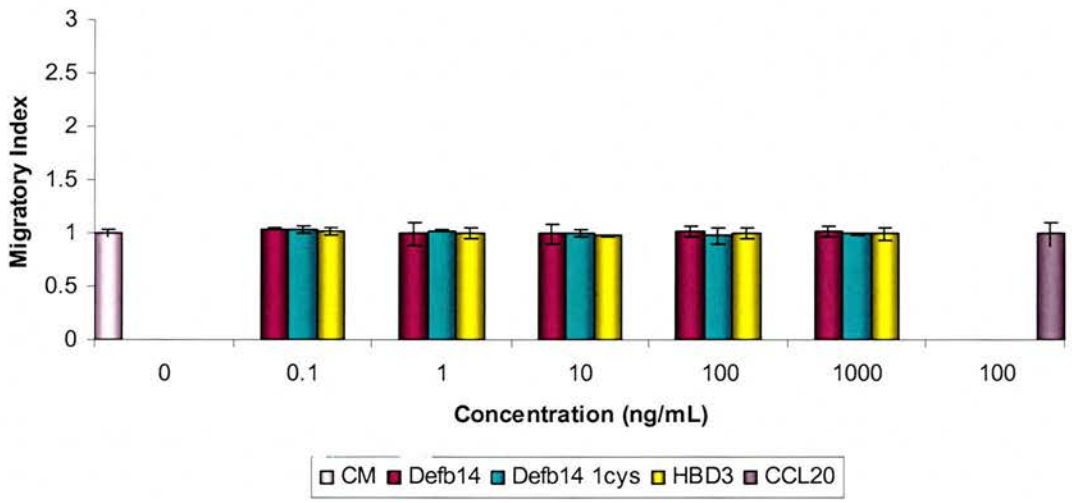


Figure 4.20 Migration of HEK293 cells in response to Defb14, Defb14 1cys and HBD3

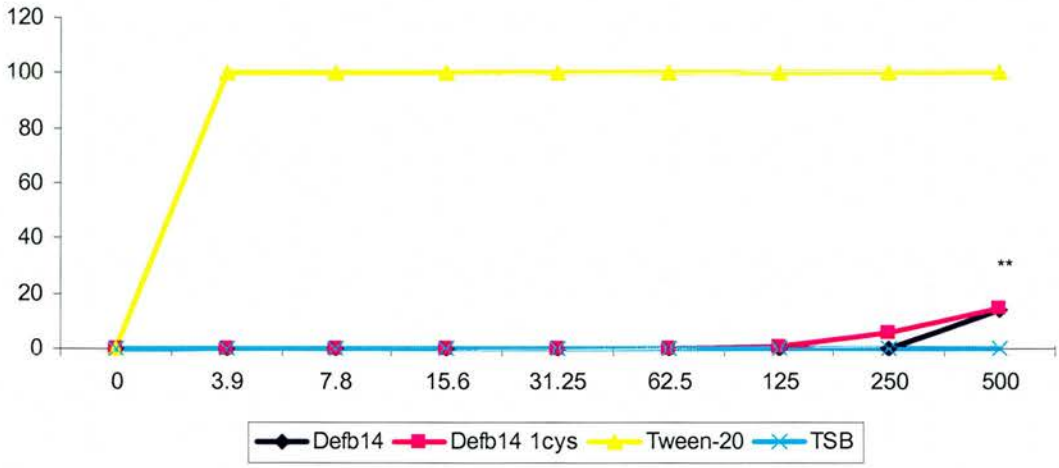
Graph A shows the mean number of cells migrated per random field of view. Graph B shown the migratory index, the number of cells migrated in each sample divided by the number of migrated cells in the media alone sample. * represents $p< 0.05$, ** $p<0.01$. Error bars represent the standard error of all nine replicate counts. Experiments were repeated three times and the above is a representative experiment (n=3).

CELL TYPE	Defb14	Defb141cys	HBD3
Monocytes (human)	1000	100	-
CD4 T cells (human)	100	10	-
Neutrophils (mouse)	No substantial migration observed	No substantial migration observed	-
CD4 T cell (mouse)	100	10	-
HEK293	No substantial migration observed	No substantial migration observed	No substantial migration observed
HEK293 CCR6	100	100	100

Figure 4.5 The chemotactic activity of Defb14, Defb14 1cys and HBD3 against various cell types

Defb14, Defb14 1 cys and HBD3 were tested against various cell types for chemotactic responses. Shown is the optimal concentration (ng/ml) that induced the maximal migration of a given cell type Not tested represented as -

A



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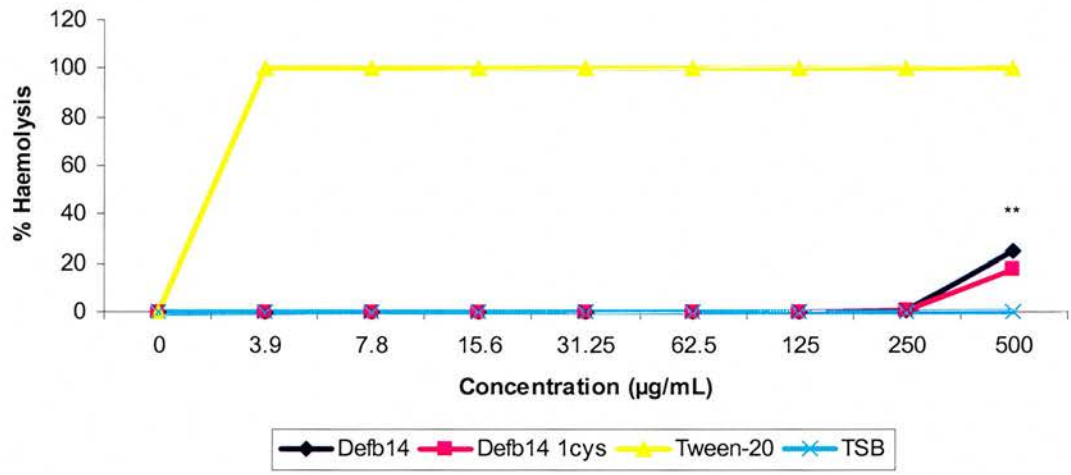


Figure 4.21 Lysis of human erythrocytes in the presence of Defb14 and Defb14 1cys +/- DTT

Graph A shows the haemolysis of human erythrocytes in the presence of Defb14 and Defb14 1 cys. Graph B shows the samples reduce with 10mM DTT
* represents $p< 0.05$, ** $p<0.01$ control vs peptide. Experiments were repeated three times and the above is a representative experiment (n=3).

4.3.12 Analyses of Defb14 all alanine

Defb14 was synthesised whereby all the cysteine residues were replaced with alanine residues (Figure 4.21). Antimicrobial analysis against *P.aeruginosa, S.aureus* and *B.cenocepacia* displayed killing activity similar to that of the Defb14 parent peptide. Interestingly the chemoattractant properties with Defb14 all alanine, were diminished when tested with HEK293 cells expressing CCR6.

A)

Defb14 all alanine

FLPKTLRKFFARIRGGRAAVLNALGKEEQIGRASNSGRKAARKKK

B)

CELL TYPE	Defb14 all alanine
HEK293	>10000
HEK293 CCR6	>10000

Values represented as ng/ml. Highest test concentration 10000 ng/ml.

C)

ORGANISM	Defb14 all alanine	Defb14
B.cenocepacia J2315	>100	>100
P.aeuriginosa PAO1	1.5	1.5
S.aureus ATCC 25923	6.25	6.25

Highest test concentration 100 μg/ml

Figure 4.22 Defb14 all alanine analyses

A) Sequence of Defb14 all alanine

B) Chemotactic activity of Defb14 all alanine

C) Antimicrobial analysis of Defb14 all alanine

4.4 DISCUSSION

Through evaluation of Defb14 the murine orthologue of HBD3 and Defb14 1cys the dimeric analogue, this study has shown the following:

- That Defb14 is expressed in a variety of murine tissues.
- That synthetic oxidised Defb14 is a mixture of monomeric species and various non-canonical disulphide connectivities.
- That this non-canonical synthetic Defb14 displayed both antimicrobial and chemoattractant properties,
- That the synthetic dimeric analogue Defb14 1cys displays antimicrobial and chemoattractant properties similar to that of the monomeric peptide.
- That Defb14 and Defb14 1cys display similar haemolytic profiles
- That both Defb14 and Defb14 1cys display salt insensitive activity regarding *P.aeruginosa.*

4.41 Sequence analysis

The evolutionary analysis of Defb14 demonstrated that this is the murine orthologue of human β-defensin 3 with 68% of the sequences being identical and only four amino acids changes that are not conservative or semi-conservative (Semple *et al.*, 2005). Genes that are involved in host defence often display high rates of genomic divergence and evidence for adaptive evolution which is thought to be a possible response to the rapid evolution of pathogens. Evolution of HBD3 does not seem to have encountered striking episodes of amino acid changes arising from positive selection although other β-defensins do show this. Interestingly of the four amino acid changes, the rat orthologue (DEFB14) has the same amino acids as the mouse at three of these positions, but the serine at position 36 in the mature peptide is a lysine in the rat and an arginine in the human. This is not a position where any change is seen in a detailed examination of the primate sequences (Boniotto *et al.*, 2003). The two N-terminal non-conservative differences between the human and mouse lie at the front of the predicted alpha helix in a sequence and 3D structure alignment with HBD3. The change between

cysteine 3 and 4 is a proline in HBD3 and a glycine in Defb14 (Table 4.1). Proline residues may be helpful in forming β-hairpins (Krishnakumari *et al.*, 2003) and is conserved in the primate HBD3 sequences.

4.42 Structural characterisations

Comparing the structural characterisation of Defb14 and Defb14 1cys was accomplished by using a variety of analytic methods. Previous structural analyses of HBD1, HBD2 and HBD3 (Schibli *et al.*, 2002) was performed using the native gel electrophoresis system that Defb14, Defb14 1cys and HBD3 have been studied with here. Using this method HBD1 and HBD2 were found to migrate on the gel with similar mobilities, whereas HBD3 ran significantly slower. According to the size of the band it can be deduced that the disulfide bonds are intact and the mobility indicates the presence of a dimer. HBD1 and HBD2 migrate at the appropriate monomer expectation. Upon reduction of the disulfide bonds with DTT, the mobility of all three of the defensins is increased and their mobilities become more similar. This potentially indicated that the tertiary structure of HBD3 is disrupted and results in a loss of the stable dimer structure when the disulfide bonds are cleaved. Defb14 1cys is a dimer due to the covalent disulfide-bridged linkage that was imposed, whereas HBD3 and Defb14 look as though they may have the ability to form dimers. However they may be forced to present a far more hydrophobic structure to the native gel via their S-S bridges which would result in a lower electrophoretic mobility. When the defensins are reduced, they are free to reorganise their polypeptide chains and the hydrophobic groups will then group together internally in the peptide structure. This will raise the mobility of the peptide through the gel and could result in the differences seen here and those reported by Schibli *et al.*, 2002.

To determine the clear oxidation states of HBD3 and Defb14 ESI-FT-ICR mass spectrometry was performed concluding that both peptides are only observed as monomers, despite their dimer like appearance on the gel. In contrast to previously studied defensins, Defr1 (Campopiano *et al.*, 2004) and Defb107 (McCullough manuscript accepted 2006) no evidence was observed for higher order non-covalent

aggregates for both Defb14 and HBD3. Defb14 and HBD3 was electrosprayed under a variety of conditions, various concentrations, pHs and buffer concentrations, however no peaks corresponding to the formation of a non-covalent dimer for either Defb14 or HBD3 was observed. This is perhaps not surprising considering the high number of basic residues contained in these peptides; columbic repulsion will dictate that they will not interact non specifically, and the findings imply that both Defb14 and HBD3 possess no particularly favoured specific interaction leading to dimerisation.

The β-defensins are often classified due to their canonical cysteine backbone. The α-defensins constitute a cysteine backbone with the connectivities cys1-cys6, cys2-cys4, cys3-cys5 (Selsted *et al.*, 1989). The typical canonical arrangement for the β-defensins is cys1-cys5, cys2-cys4, cys3-cys6. With Defb14 no evidence was seen for this β-defensin bridging pattern. The dominant connectivities observed of cys1-cys2, cys3-cys4, cys5-cys6 and cys1-cys4, cys2-cys5 and cys3-cys6 are previously undescribed arrangements for defensins and will present an extended and disordered conformation (confirmed by NMR analysis). The sterically unlikely cys5-cys6 bond has previously been described in hepcidin, a four-disulfide amphipathic cationic peptide made in the liver where it is believed to have functional significance (Hunter *et al.*, 2002). HBD3 does show some evidence for a canonical β-defensin fold. For both Defb14 and HBD3 proteolytic digestion produced several unidentified peaks, and it is possible that some conformers with connectivities other than those referred to in Table 4.2 are present in lower abundancies. Kluver *et al.*, 2005, synthesised two HBD3 peptides with the disulfide pattern predicted for β-defensins and resulted in significantly different products. From the oxidative folding of the 45 residue peptide a major product with the desired disulfide connectivity was found. The second peptide, a 40 residue with the first five residues missing from the mature peptide sequence led to a mixture of fully disulfide bonded isomers containing no significant amounts of the product with the β-defensin disulfides. Since both were performed under identical reaction conditions it is possible that the first five residues of the peptide facilitate the formation of the β-defensin specific disulfide bonded pattern and are important sterically in the formation

of a key intermediate. Although Wu et al., 2003 found that HBD3 did not fold preferentially into a native conformation *in vitro* under various oxidative conditions.

Three dimensional structures of a number of defensins have been determined by both NMR and x-ray crystallography (White et al., 1995). Regarding human β-defensins the first structure for HBD2 was reported using NMR (Schibli *et al.*, 2002) and also for x-ray crystallography (Hoover *et al.*, 2000). Previous HBD3 analysis by Schibli *et al* 2002, was found to be in agreement with our preparation of HBD3. Signals at both extremes of the HBD3 spectrum are in agreement with the reported chemical shifts for canonically folded HBD3. HBD3 has been suggested to form a stable dimer through an intermolecular salt bridge between Glu^{28} on one monomer and Lys^{32} on another via a symmetrical H-bond interaction between Gln^{29} of both monomers. The NMR work of Schibli *et al* 2002 did not directly show this. This dimer formation will also rely on intramolecular bridging between Glu^{27} and Arg^{17}. The role of these residues was recently explored by the study of a triple mutant, which replaced this putative dimerisation motif. The mutant HBD3 was a monomer but found to be just as active as the wild type HBD3 (Boniotto *et al.*, 2003). Three of the four sites are conserved between human and mouse, but the fourth, Lys^{32} has semi-conservatively changed in the mouse sequence to an arginine. Analysis with ESI-MS revealed that there is no evidence for dimer formation for HBD3 although its identifiable S-S connectivities are consistent with β-defensins. Schibli *et al.*, 2002 also reported that HBD3 does not appear as a dimer via ESI-MS although they do not specify the conditions employed. This result is contrary to the experimental results obtained in solution by diffusion based experiments (NMR) and light scattering which indicates that HBD3 is a dimer.

4.43 Antimicrobial analysis

Previous antimicrobial activity analysis of a five cysteine unique murine defensin related peptide (Defr1) displayed antimicrobial activity in its dimeric form. This activity was lost upon reduction of the peptide to a monomeric form (Campopiano *et al.*, 2004). In this specific case it was concluded that the antimicrobial activity of Defr1 was directly related to its tertiary structure. With Defb14 antimicrobial activity assays were

performed against a panel of Gram positive and Gram negative organisms. The six cysteine murine orthologue of HBD3 revealed that its activity is comparable with that reported for HBD3 (Harder *et al.*, 2001, Wu *et al.*, 2003, Boniotto *et al.*, 2003). This activity is not altered upon reduction with DTT and is therefore independent of disulfide structure. When analysing the single cysteine dimeric analogue Defb14 1cys, this displayed the same minimum bactericidal concentrations (MBC) values as the original Defb14. This activity is not lost when the dimer is reduced to a monomer using DTT. The Defb14 all alanine peptide was also shown to be antimicrobial yet contained no cysteine residues. Despite synthetic Defb14 having a disordered structure with a mix of novel disulfide connectivities it is still an effective antimicrobial and comparable with the human orthologue HBD3. This work contradicts the Defr1 study but is in agreement with Wu *et al.*, 2003 the cysteine bonding is not relevant to this activity. Further studies to validate the irrelevance of cysteines for antimicrobial activities include a completely linear preparation of synthetic HBD3 (Hoover *et al.*, 2003). All the cysteine residues were removed and replaced with α-aminobutyric acid creating a peptide which showed the highest activity against *E.coli* and *S.aureus*.

The antimicrobial activity of Defb14 and Defb14 1cys was analysed in the presence of 0.1 %, 1 % and 10 % serum. Both peptides displayed killing under these conditions although at 10% serum 100% killing was unattainable. Several studies have displayed an activity decreasing effect with the presence of divalent cations (Tomita *et al.*, 2000, Hoover *et al.*, 2003, Kluver *et al.*, 2005). The divalent cations are thought to compete with the peptides for the binding sites on the negatively charged bacterial cell wall therefore only peptides with a higher net charge can access the cell surface. The antimicrobial activity of both Defb14 and Defb14 1 cys was evaluated at various salt concentrations. Defb14 and Defb14 1cys remained unaffected when tested in this range against *P.aeruginosa* PAO1. When tested against *S.aureus* ATCC 25923 in this assay a higher concentration of the peptides was required for killing at 250mM. Harder *et al.*, 2001 tested HBD3 against *S.aureus* ATCC 6538 and concluded that activity declined in the presence of 250 mM NaCl. HBD3 has also been tested in the presence of 100 mM and 500 mM NaCl against *A.actinomycetemcomitans* and *S.mutans*. At 100 mM both

these organisms remained unaffected when tested with HBD3. In contrast the ability of HBD1 and HBD2 to kill was reduced (Ouhara et al., 2005). In 500 mM NaCl all the peptides showed inhibition of antibacterial activity.

4.4.5 Chemotactic analysis

Chemotactic activity has been displayed in a variety of defensins including HBD3. Activity has been detected against human monocytes at a migratory index of 2.0 (Garcia *et al.*, 2001a). Chemotactic activity has also been identified with immature dendritic cells (Yang *et al.*, 2002) but not with mature dendritic cells. The chemotactic activity of HBD1, HBD2 and HBD3 for immature dendritic cells is mediated by the chemokine receptor CCR6 (Yang *et al.*, 1999). The chemotactic activity of HBD3 to monocytes must use another receptor other than CCR6 because monocytes do not express functional CCR6. The monomeric Defb14 displays poor activity against monocytes. This is perhaps expected because the preparation appeared to be a mix of analogues all without the canonical disulfide binding. The Defb14 1cys activity against monocytes (optimal concentration 100ng/ml) is as potent as that reported for the canonical form of HBD3 (Wu *et al.*, 2003) despite being a mouse derived peptide interacting with a human receptor. This implies that the structure directed by intramolecular S-S binding is not essential for the defensin induced chemotactic response of monocytes. Both Defb14 and Defb14 1cys are equally active against HEK293 cells expressing CCR6 and this activity is equivalent to that of our synthetic canonically bound HBD3 and is equivalent and equal to that which we observe with CCL20/MIP-3α (the only chemokine known to interact with the CCR6 (Schutyser *et al.*, 2003). This again implies that the canonical bonding is not essential for the chemotactic activity for Defb14 although the Defb14 all alanine peptide displayed no chemotactic properties against HEK293 CCR6. Comparison of the crystal structure of CCL20 and HBD1 and HBD2 has revealed some structural similarities despite no sequence similarity (Hoover *et al.*,2002). An Asp^4-Leu^9 motif in HBD2, which resembles the Asp^5-Leu^8 motif of CCL20/MIP-3α is considered to be responsible for specific interaction with CCR6, providing a structural basis for the capacity of β-defensins and

CCL20/MIP-3α to interact with the same receptor. This region flanks the first cysteine and yet Defb14 1cys without a cysteine at this first position is still active in attracting CCR6 expressing cells.

4.45 Haemolytic profile

The interaction of β-defensins with eukaryotic membranes is not well understood. HBD3 has been shown not to exhibit significant lytic activity on human erythrocytes (Harder *et al.*, 2001) between 0 - 500 μg/ml. Defb14 and Defb14 1cys gave similar profiles against human erythrocytes with no significant lytic activity observed. Generally it is assumed that the larger the number of hydrophobic amino acids leads to an increased haemolytic activity with erythrocytes. Eukaryotic cell membranes consist mainly of zwitterionic phosphatidylcholine and phosphatidylethanlamine susceptible to hydrophobic interactions (Yeaman *et al.*, 2003). Kluver *et al* 2005 described differences in sequence of the HBD3 peptide responsible for haemolysis rather than the disulfides.

In summary, these results highlight that despite synthetic oxidised Defb14 being a mixture of monomeric species and various non-canonical disulfide connectivities, antimicrobial and chemotactic properties are still observed. In addition the dimeric analogue with only one cysteine residue displays equivalent antimicrobial and chemotactic activity as the six cysteine parent Defb14.

CHAPTER 5:

Defb14 INSPIRED PEPTIDES

5.1 INTRODUCTION

During the past century antimicrobial agents have become a necessity in combating infections previously considered life threatening. However the past decade has seen infections considered curable returning with the ability to defy established antimicrobials. Existing established agents have provided accurate protection and often act as a template for the development of analogues within the family. Improvements in efficacy, solubility, toxicity and cost have contributed to the success and exploitation of these properties.

Established antimicrobials have been modified in an attempt to optimise the desirable properties and importantly evade problems with resistance. Previously it was very common to generate active analogues of existing antimicrobials in hope that there would always be a backup ready if problems were encountered. Unfortunately this has become increasingly difficult, few antimicrobials are being approved and new classes are virtually unheard of. Recently tigecycline, a member of a new class of antimicrobial agents, the glycyclines, was approved for treatment (Bosso *et al.*, 2005) against complicated skin infections. The glycyclines are derivatives of the tetracycline and overcome the existing problems that these agents have previously encountered (Fraise *et al.*, 2006) with resistance.

The use of peptides of the innate immune system as therapeutic anti-infectives has attracted a lot of commercial research. Various companies have been established to pursue a new generation of peptide antimicrobials. Intrabiotics Inc was based on the discovery of porcine leukocyte protegrins (Kokryakov *et al.,* 1993) and their ability to exhibit broad spectrum antimicrobial activity, low emergence of resistance and the ability to maintain their activity in saliva. Disappointingly these peptides failed to display any clinical advantage at Phase III trials. Helix Biomedix Inc focuses on a diverse array of peptides including the polyphemusins, cecropins and melittins. They aim to exploit the both the antimicrobial and modulatory properties of these peptides. Current successes include potential therapeutic peptides HBCM2 and HBCM3 which display potent *in vitro* and *in vivo* activity (Zhang *et al.*, 2005). Micrologix Biotech Inc has developed analogues to indolicidin isolated from bovine neutrophils (Selsted *et al.*,

1992). Efforts have been concentrated on the development of topical application for skin disorders and infections. Recently the discovery of plectasin, the first defensin to be isolated from a fungus *Pseudoplectania nigrella* (Mygind *et al.*, 2005) by Novazymes has been encouraging. Plectasin displays extremely low toxicity in mice and has cured peritonitis and pneumonia caused by *S.pneumoniae* as efficiently as penicillin and vancomycin.

The antimicrobial peptide families are ideal candidates for therapeuctic agents and functional derivatives have displayed attractive properties. Derivatives of dermaseptin S4 have displayed improved toxicity profiles and desirable antimicrobial values by altering the composition of hydrophobic domains, reducing the hydrophobicity and by increasing the net positive charge (Navon-Venezia *et al.*, 2002). Completely synthetic peptides, similar to that of naturally occurring peptides like tracheal antimicrobial peptide, defensins and magainins have been studied for their activity against multidrug-resistant cystic fibrosis pathogens (Schwab *et al.*, 1999). Under optimal conditions these peptides have been shown to be highly potent against multi-drug resistant pathogens, including mucoid *P.aeruginosa* and MRSA. Libraries of LL-37 derived synthetic peptides have been generated and their anti-infective properties and host stimulatory properties were evaluated (Braff *et al.*, 2005). Synthetic peptides with the first six and seven residues missing from the complete LL-37 gave lower MIC values against the panel of organisms tested.

Regarding the β-defensins active analogues have been generated from the sequence of HBD1, HBD2 and HBD3 (Krishnakumari *et al.*, 2006). These peptides, based on the C-terminus of the original defensins have displayed activity against *E.coli* (15-17 μM) and *S.aureus* (17-20 μM). HBD3 has been subject to several derivative studies (Wu *et al.*, 2003, Hoover *et al.*, 2003 and Kluver *et al.,* 2005) with various regions of the peptide being of interest. Synthetic peptides have been generated at both the N-terminus and C-terminus of HBD3. Variations in the number of residues, the overall net charge and the hydrophobicity have been studied for their impact on antimicrobial activity.

This chapter describes the first documented study involving synthetic Defb14 fragments. Peptide fragments were prepared and analysed to aid in identifying antimicrobially active sequences. Further to this, experiments were performed for functional analyses including chemotaxis, haemolysis and salt sensitivity.

5.2 AIMS

The aims of this chapter were to:

1 Determine the activity of synthetic Defb14 fragments

2 Determine whether the antimicrobial activity could be attributed to a specific region of the mature Defb14 peptide

3 Evaluate the activity of the Defb14 fragments as chemoattractants

4 Evaluate the haemolytic and salt sensitivity profiles of the Defb14 fragments

5.3 RESULTS

Synthetic fragments of Defb14 were prepared and analysed to elucidate their antimicrobial and chemoattractant properties. Defb14 fragments were created based on the original sequence with the cysteine residues replaced with alanine residues (Maemoto *et al.*, 2004). In total seven fragments were created and analysed for functionally active sequences.

5.3.1 Synthetic preparations of Defb14 fragments

Synthetic Defb14 fragments were created in an attempt to locate functionally active regions associated with the Defb14 sequence. Initially the Defb14 all alanine peptide sequence was halved and both the N-terminus and C-terminus fragments were created (Figure 5.1). A further change to the C-terminus involved replacing one alanine residue with a cysteine residue to enable analyses of a potential dimeric formation. Subsequent analyses concentrated on the N-terminus with a further three fragments created to identify residues essential for antimicrobial activity. In addition a fragment which overlapped both the N-terminal and C-terminal half was synthesised. The net charge of the fragments ranged from +6 to +2 with the original Defb14 peptide being +12. The hydrophobicities of the fragments ranged from -1.62 to -12.35 with the original Defb14 at -12.65 (Table 5.1).

5.3.2 Antimicrobial analysis of Defensin inspired peptides 1-7

Antimicrobial analysis was performed on all the Defb14 inspired peptide fragments against a panel of Gram positive and Gram negative organisms. Dip 1, 4 and 5 display potent antimicrobial activity against a spectrum of organisms (Figure 5.2-5.9). Interestingly the initial N-terminal fragment of Defb14, Dip 1 gave potent antimicrobial activity with MBC values of 3.13 μg/ml ($p<0.01$), 1.5 μg/ml ($p<0.01$) and 3.13 μg/ml ($p<0.01$) against *E.coli*, *P.aeruginosa* and *S.aureus* (Table 5.2). These values are identical to those of the Defb14 parent sequence. Analysis of the C-terminus with Dip 2 and 3, concluded poor activity with MBC values greater than 50 μg/ml. Dip 3 included

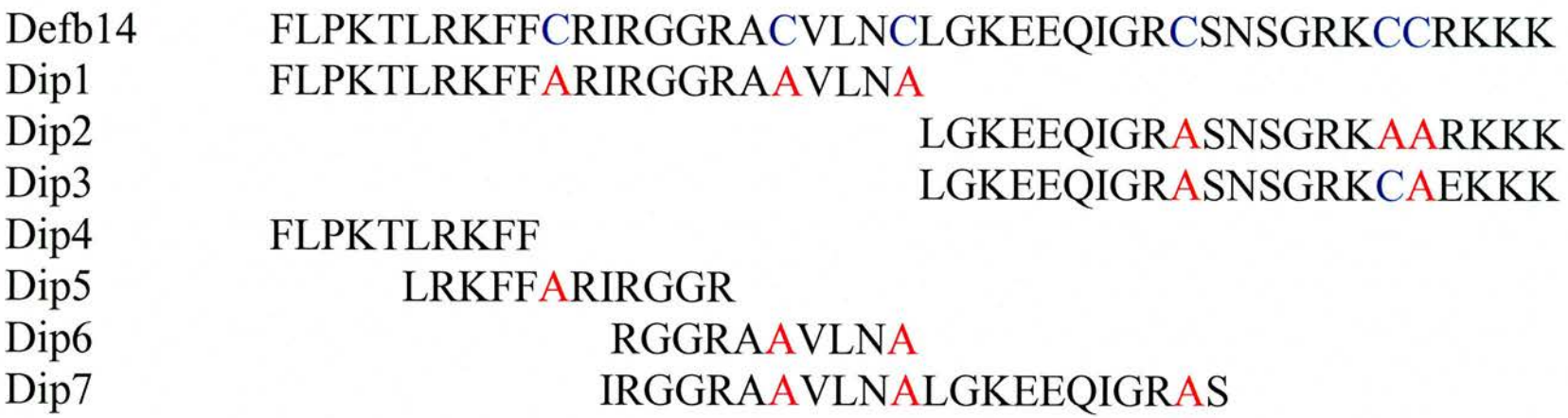


Figure 5.1 Defb14 and Defb14 inspired peptides

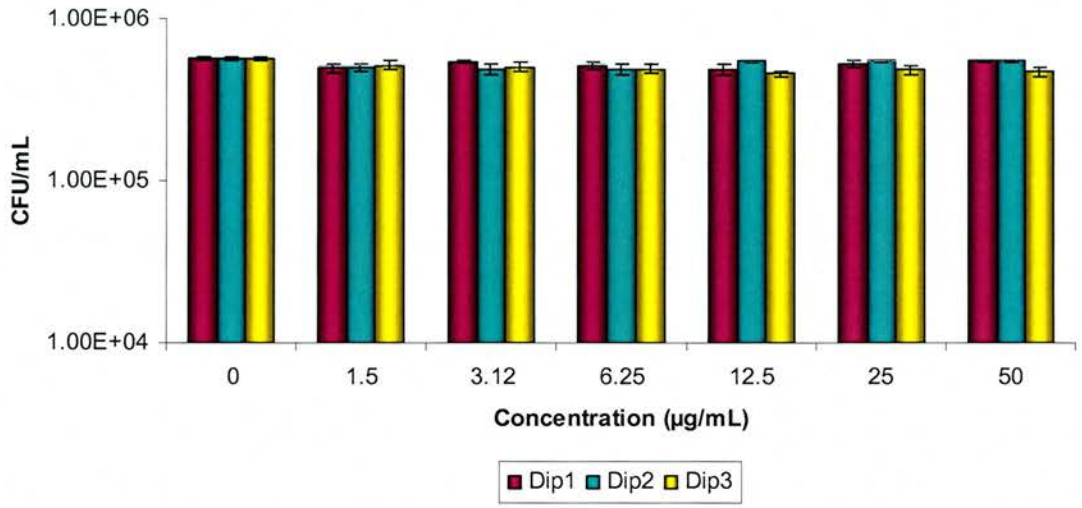
The above figure represents the sequences of the Defb14 inspired peptides and their position in relation to the full length Defb14. Amino acids highlighted in blue indicate the conserved cysteine residues and those highlighted in red denote the changes from cysteine to alanine

PEPTIDE	SEQUENCE	+	HYDROPHOBICITY (ΔG)
Dip1	FLPKTLRKFFARIRGGRAAVLNA	+6	-1.62
Dip2	LGKEEQIGRASNSGRKAARKKK	+6	-12.35
Dip3	LGKEEQIGRASNSGRKCARKKK	+6	-11.94
Dip4	FLPKTLRKFF	+3	1.13
Dip5	LRKFFARIRGGR	+5	-1.29
Dip6	RGGRAAVLNA	+2	-2.08
Dip7	IRGGRAAVLNALGKEEQIGRAS	+2	-7.64

Table 5.1 Sequence, charge and hydrophobicities of Defb14 inspired peptides
(http:/aps.unmc.edu/AP/main.php)-Antimicrobial Peptide Database

The peptide sequence, charge and hydrophobicities of Defb14 inspired peptides are shown in the above table.
Amino acids highlighted in blue indicate the conserved cysteine residues and those highlighted in red denote the changes from cysteine to alanine. ΔG = hydrophobicity score in water of monomeric peptides calculated using Wimley and White scale, greater hydrophobicity indicated by a less negative value.
+ indicated net charge of monomer
Peptides supplied by D.MacMillan

A



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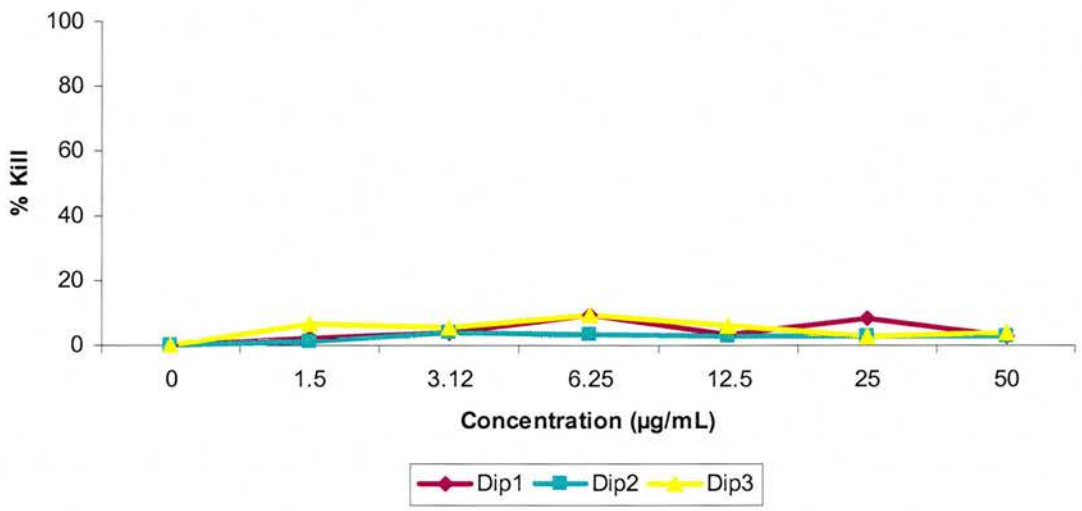
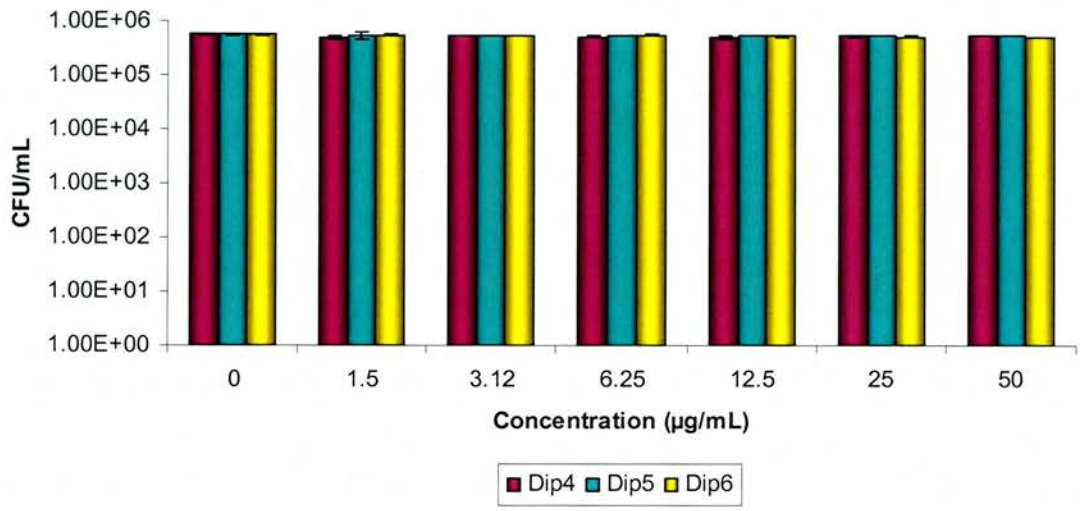


Figure 5.2 Antimicrobial activity of Dip 1, 2 & 3 against *B.cenocepacia* J2315

Graph A shows the number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

A



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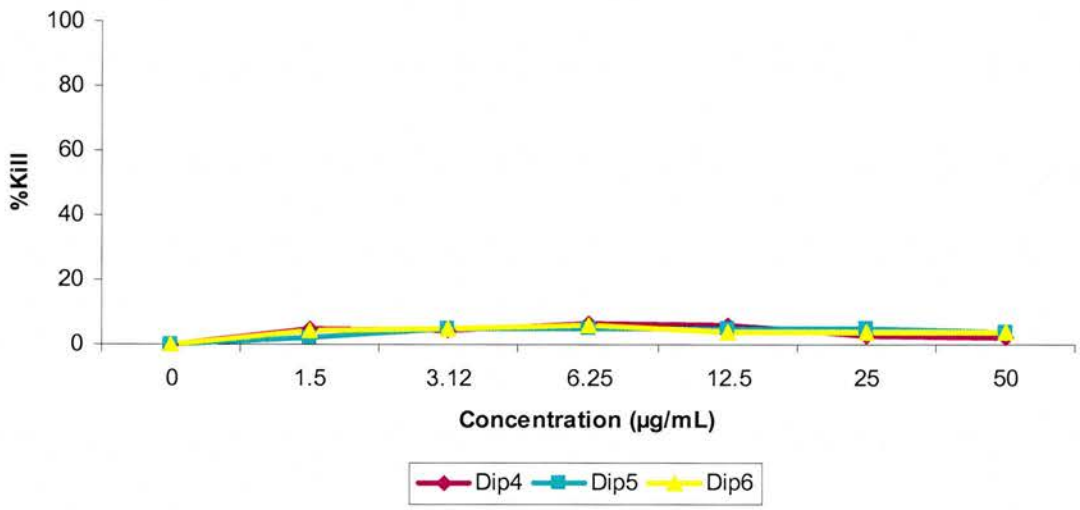
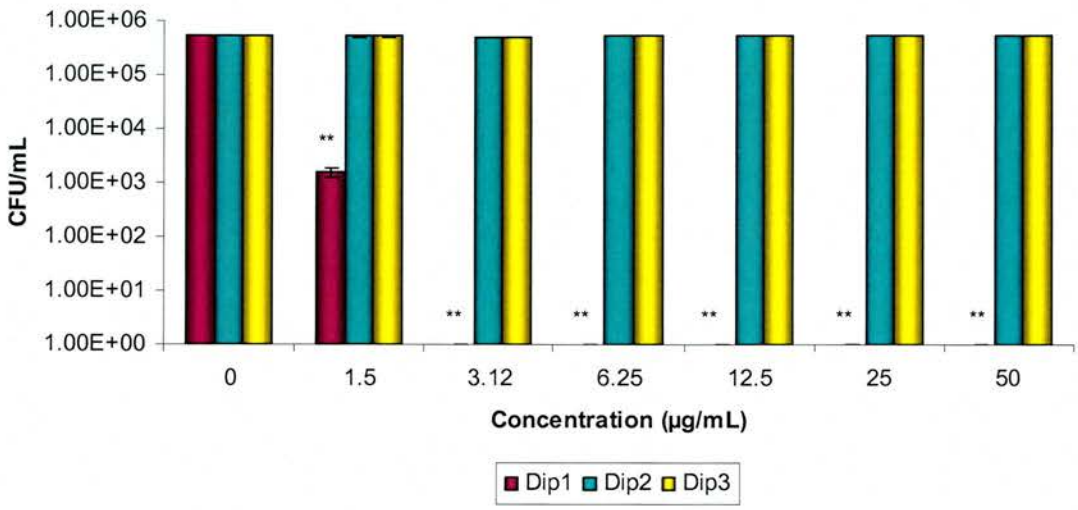


Figure 5.3 Antimicrobial activity of Dip 4, 5 & 6 against *B.cenocepacia* J2315

Graph A shows the number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

A



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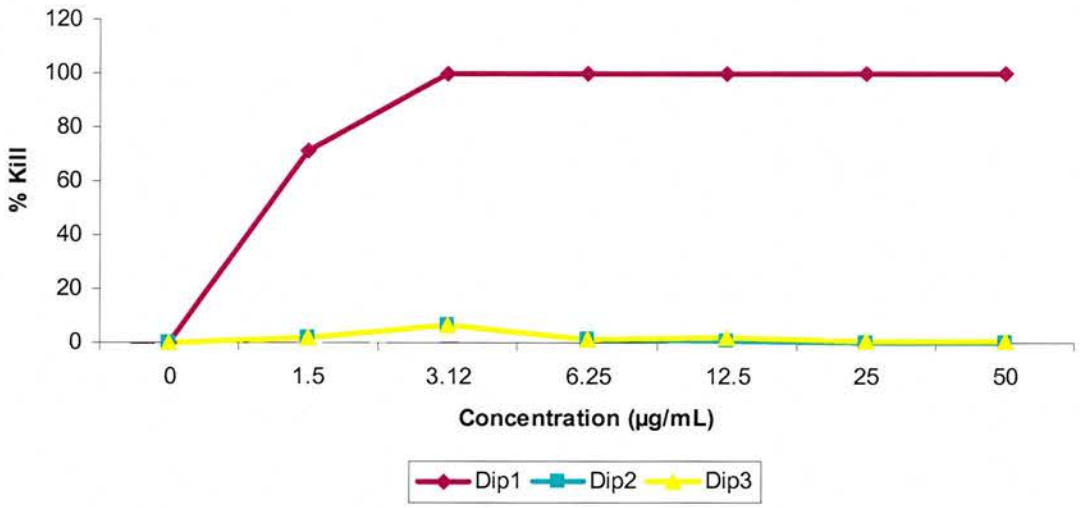
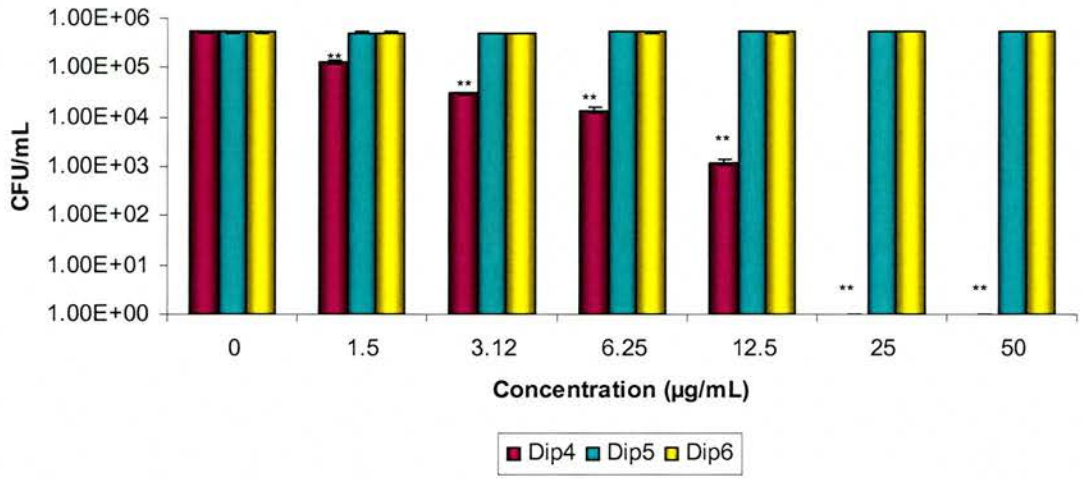


Figure 5.4 Antimicrobial activity of Dip 1, 2 & 3 against *E.coli* ATCC 25922

Graph A shows the number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

A



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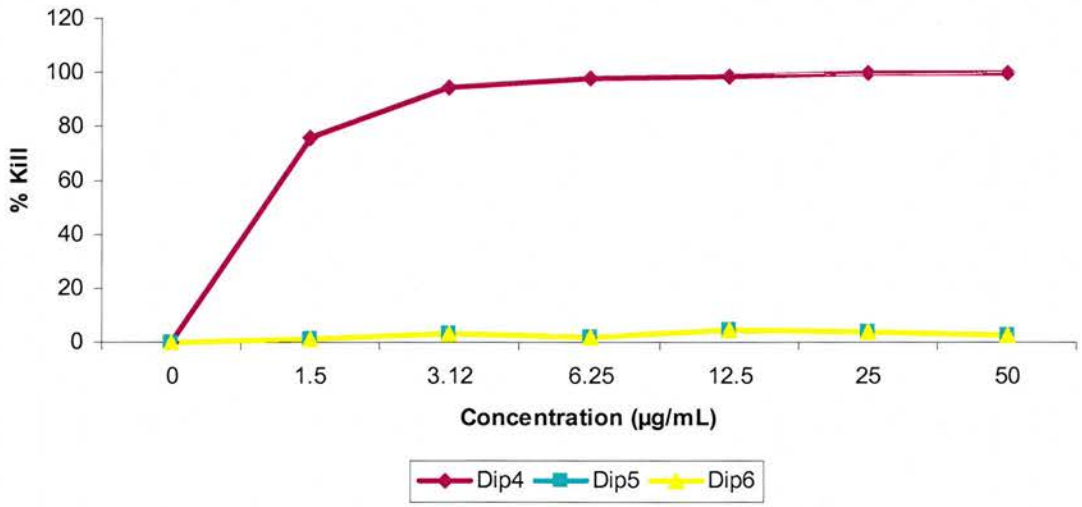
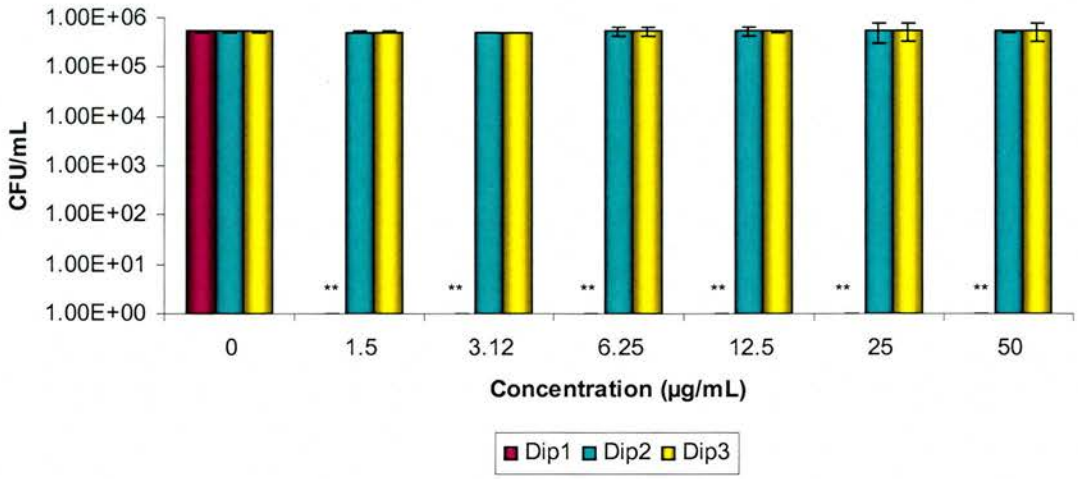


Figure 5.5 Antimicrobial activity of Dip 4, 5 & 6 against *E.faecalis* ATCC 700802

Graph A shows the number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

A



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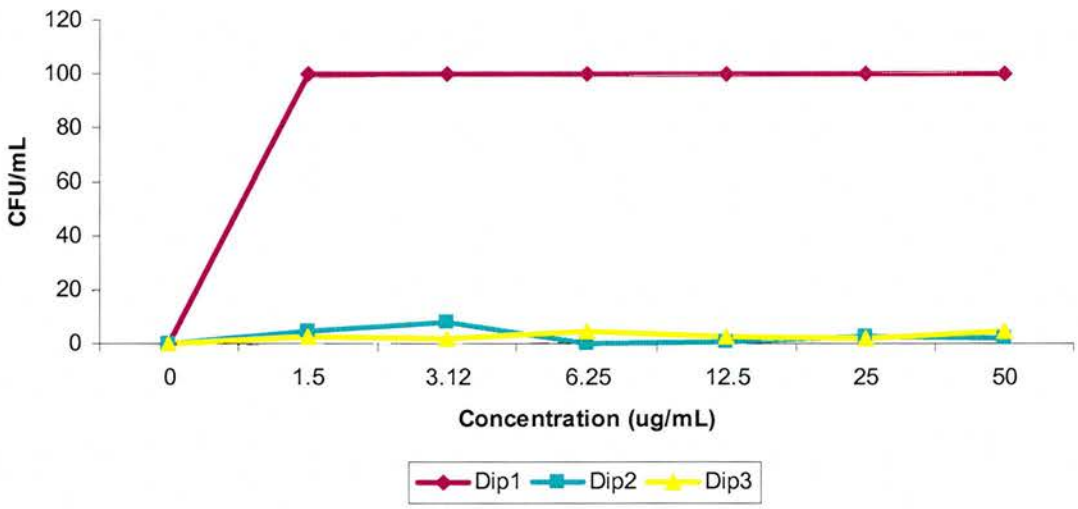

Figure 5.6 Antimicrobial activity of Dip 1, 2 & 3 against *P.aeruginosa* PAO1

Graph A shows the number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

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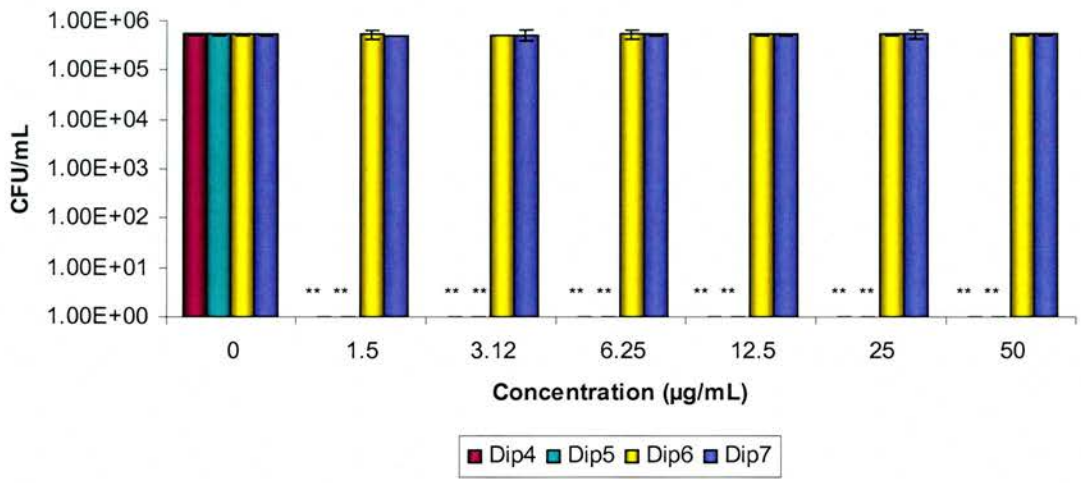

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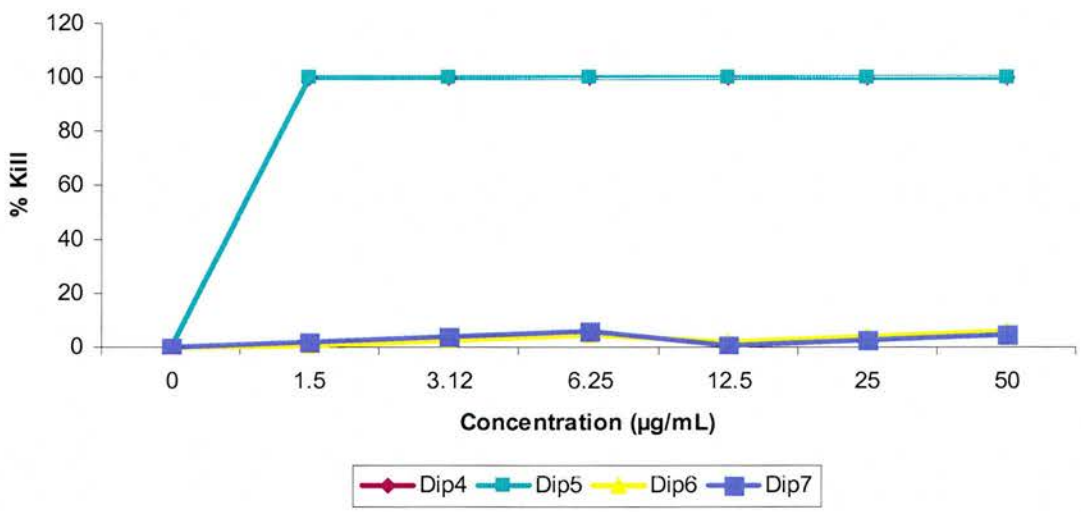
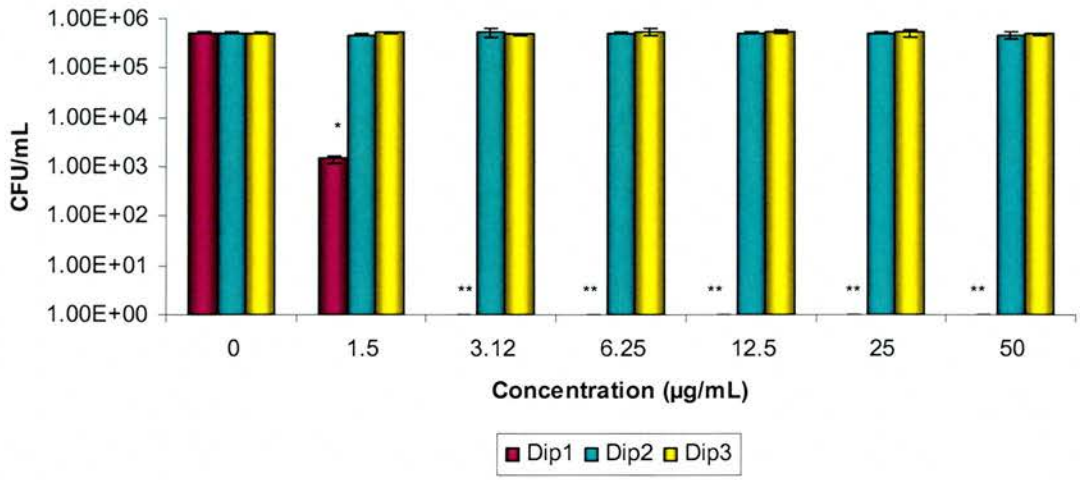


Figure 5.7 Antimicrobial activity of Dip 4, 5, 6 & 7 against *P.aeruginosa* PAO1

Graph A shows the number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

A



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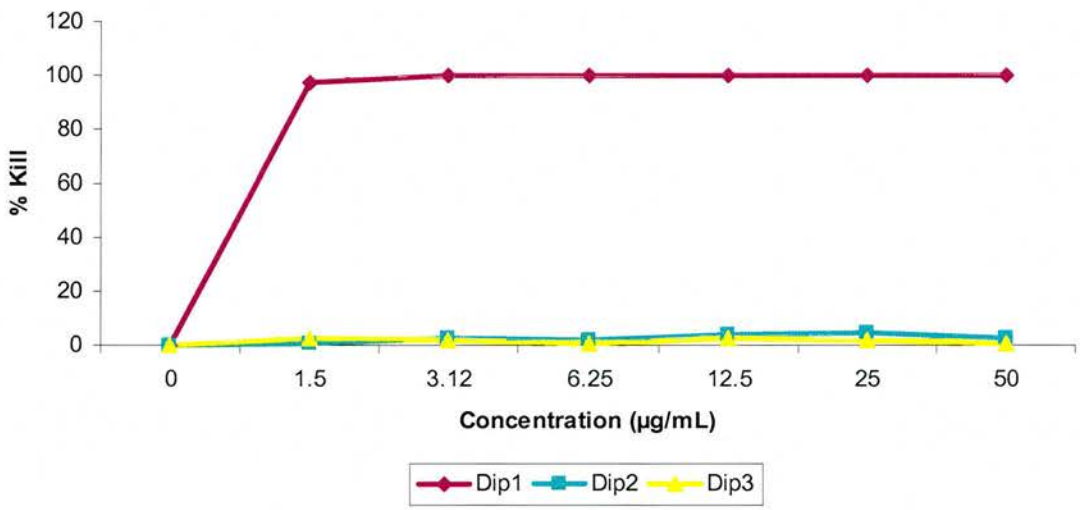
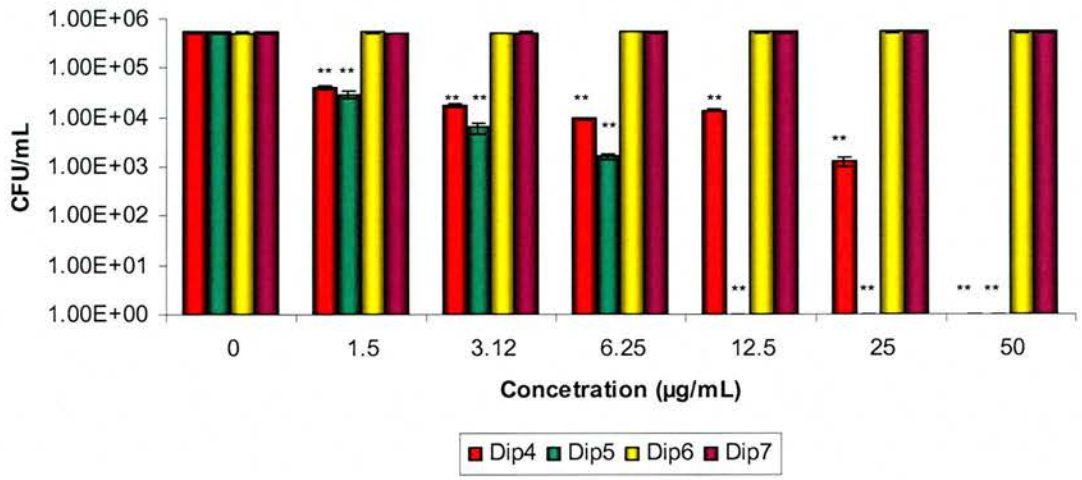


Figure 5.8 Antimicrobial activity of Dip 1, 2 & 3 against *S.aureus* ATCC 25923

Graph A shows the number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

A



B

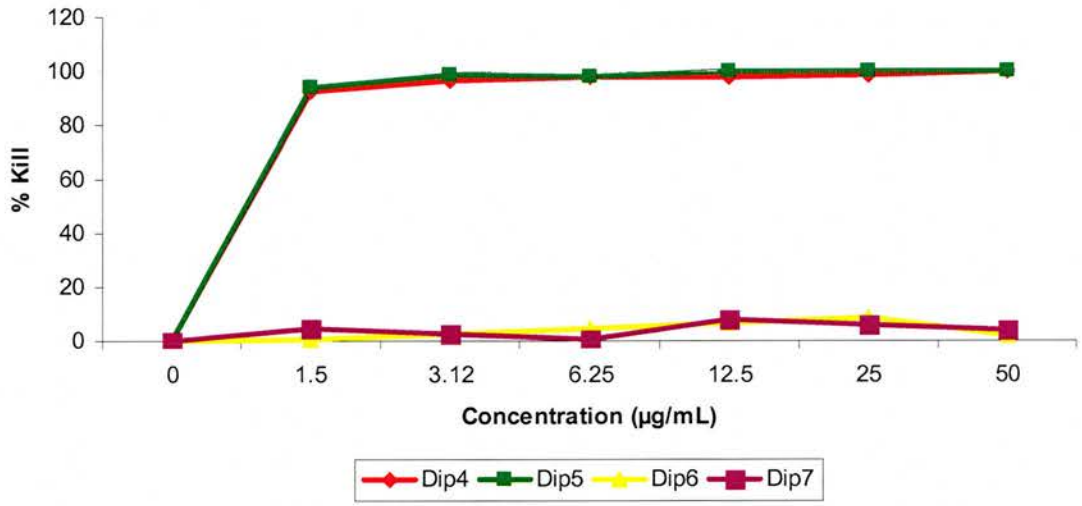


Figure 5.9 Antimicrobial activity of Dip 4, 5, 6 & 7 against *S.aureus* ATCC 25923

Graph A shows the number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples. Graph B shows the mean percentage kill; this is assessed by calculating the number of CFU surviving in the peptide treated sample as a percentage of the counts from the blank control sample. ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

ORGANISM	Dip1	Dip2	Dip3	Dip4	Dip5	Dip6	Dip7	Defb14
B. cenocepacia J2315	>50	>50	>50	>50	>50	>50	-	>100
E. coli ATCC 25922	3.13	>50	>50	-	-	-	-	3.13
E. faecalis ATCC 700802	-	-	-	25	25	>50	-	6.25
P. aeruginosa PAO1	1.5	>50	>50	1.5	1.5	>50	>50	1.5
S. aureus ATCC 25923	3.13	>50	>50	50	12	>50	>50	3.13

Table 5.2 Minimum bactericidal values of Defb14 and Defb14 inspired peptides

Defb14 and Defb14 inspired peptides were tested for antimicrobial properties against a panel of microorganisms. Minimum Bactericidal Concentrations were presented as μg/ml.
Not tested represented as -

a change from an alanine to a cysteine at position 5 (of the original Defb14 sequence). Both the C-terminal peptides Dip 2 and 3 gave similar values. Further analysis on the N-terminus to identify antimicrobially active sequences was conducted (Table 5.2). Dip 4, 5 and 6 were synthesised to focus on the N-terminal half. Dip 4 and 5 gave similar values, 25 μg/ml ($p<0.01$), and 1.5 μg/ml ($p<0.01$) against *E. faecalis* and *P.aeruginosa*. An antimicrobial difference was detected with *S.aureus* with Dip 4 giving 50 μg/ml ($p<0.01$) and Dip 5 at 12 μg/ml ($p<0.01$). Dip 6 displayed no MBC values at concentrations less than 50 μg/ml against any of the organisms tested. This peptide focuses on the peptide sequence furthest from the N-terminus but still retained within the first half of the peptide. The sequence of Dip 7 was created to analyse an overlap of both the N-terminal and C-terminal half. This peptide gave no MBC value below 50 μg/ml against *P.aeruginosa* and *S.aureus*. However Dip7 carries a net charge of +2 and hydrophobicity of -7.64.

5.3.3 Evaluation of varying salt concentrations on antimicrobial activity

To identify the effect of NaCl on the antimicrobial activity of Dip 1, 4, and 5 varying salt concentrations were evaluated (Table 5.3). *P.aeruginosa* was tested against these peptides in the presence of 50 mM to 250 mM concentration of NaCl. All Dips tested gave MBC values at 1.5μg/ml against *P.aeruginosa* in varying salt concentrations. No changes in MBC values were detected from any of the Dips at any of the salt concentrations. The values remained the same.

5.3.4 Evaluation of varying human serum concentrations on antimicrobial activity

To identify the effect of human serum on the antimicrobial values of Dip1 varying concentrations of serum were evaluated against *P.aeruginosa PAO1* (Figure 5.10). Pooled human serum was used at concentrations 0.1 %, 1 % and 10 %. Dip 1 still maintained its antimicrobial properties within the presence of serum (0.1 % ($p<0.01$), 1 % ($p<0.01$) and 10 % $p<0.01$) compared to the 0 % serum free control.. At 10 % serum complete killing was not observed. Dip 1 was tested at 50 μg/mL.

NaCl (mM)	Dip 1	Dip 4	Dip 5
0	1.5	1.5	1.5
50	1.5	1.5	1.5
100	1.5	1.5	1.5
150	1.5	1.5	1.5
200	1.5	1.5	1.5
250	1.5	1.5	1.5

Table 5.3 Antimicrobial analysis of Dip 1, 4 and 5 against *P. aeruginosa* PAO1 in various salt conditions

The antimicrobial properties of Dip 1, 4 & 5 were evaluated against *P. aeruginosa* PAO1 in varying NaCl conditions. Minimum Bactericidal Concentrations were presented as µg/ml.

A

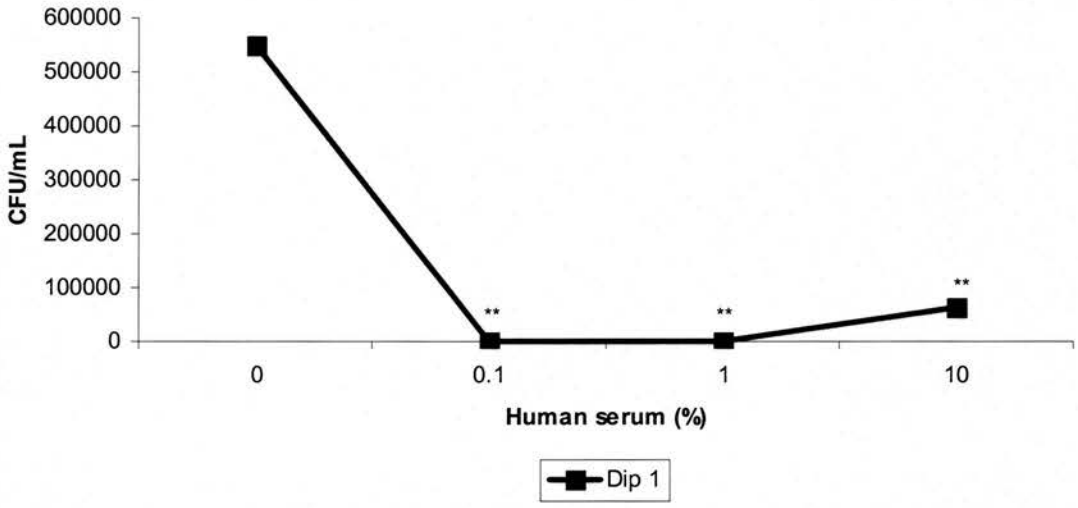


Figure 5.10 Antimicrobial activity of Dip 1 against *P.aeruginosa PAO1* in the presence of human serum

Bacteria were incubated with the peptide at 50 μg/mL over a range of human serum percentages. Graph A shows the number of colony forming units (CFU) ± standard error from the blank control and the peptide treated samples ** = $p<0.01$, * = $p<0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

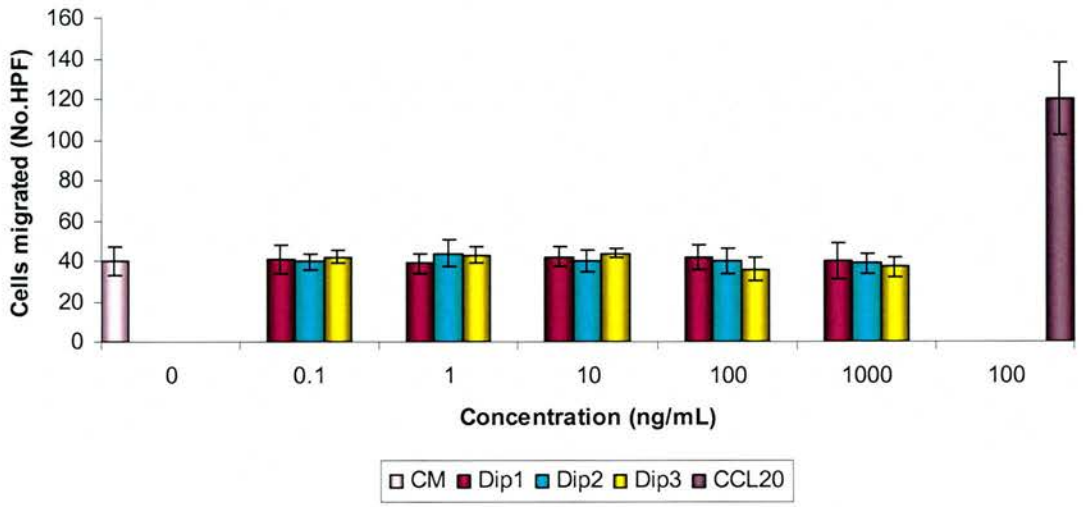
5.3.5 Chemoattractant analysis of Defb14 inspired peptide fragments

The chemoattractant properties of Dip 1, 2 and 3 were evaluated using HEK293 cells expressing CCR6 (Figure 5.11). No migration was observed from any of the Dips tested at the varying concentrations. The control chemokine, CCL20 / MIP3α induced migration at around 120 cells. The Dips concluded no migratory properties at the concentrations tested against this cell type.

5.3.6 Hemolytic activity of Defb14 inspired peptides fragments

The haemolytic activity of Dip 1, 2 and 3 were evaluated against human erythrocytes in (Figure 5.12). A range of concentrations from 3.9 μg/ml to 500 μg/ml were tested for all Dip 1, 2 and 3 all giving similar values. At the highest concentration 500 μg/ml, Dip 1, 2 and 3 displayed around 20 % haemolysis ($p<0.01$).

A



B

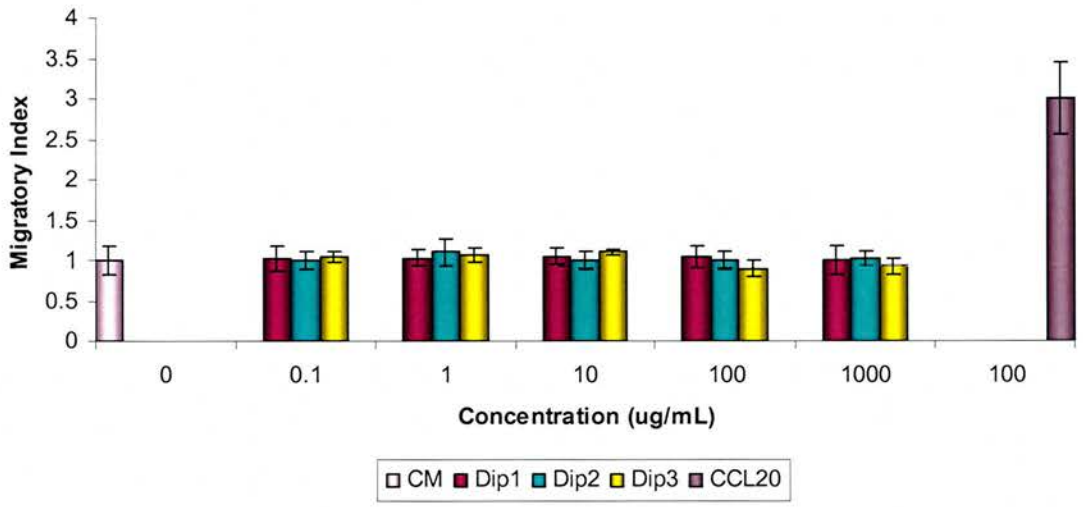


Figure 5.11 Migration of CCR6 expressing HEK293 cells in response to Dip 1, 2 & 3

Graph A shows the mean number of cells migrated per field of view from a total of nine random fields. Graph B shows the migratory index, the number of cells migrated in each sample divided by the number of migrated cells in the media alone sample. * represents $p<0.05$, ** $p<0.01$. Error bars represent the standard error of all nine replicate counts. Experiments were repeated three times and the above is a representative experiment (n=3).

A

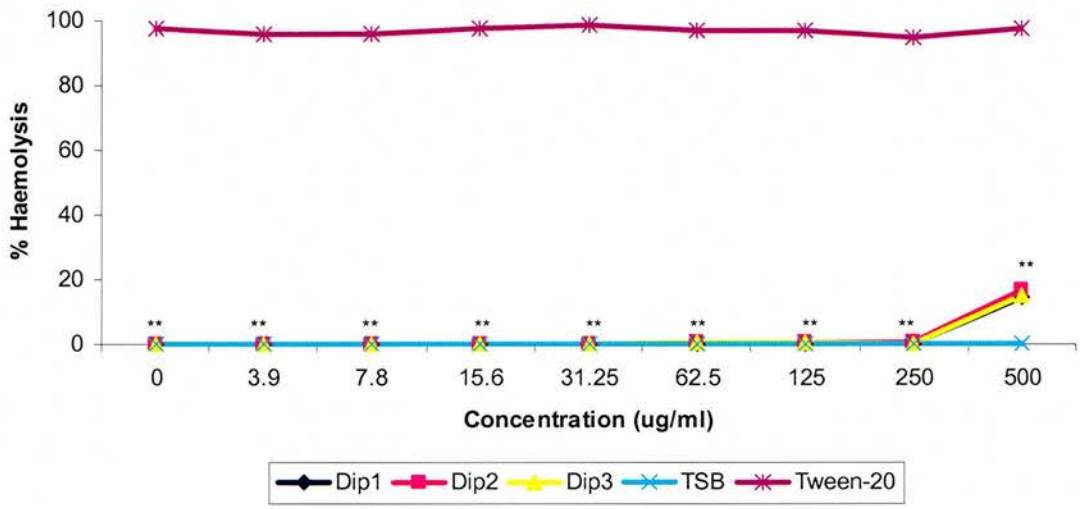


Figure 5.12 Lysis of human erythrocytes in the presence of Dip 1, 2 & 3

Graph A shows the haemolysis of human erythrocytes in the presence of Dip 1, 2 and 3. Tween-20 positive control, TSB negative control. ** = $p<0.01$, * = $p<0.05$ control vs peptide. Experiments were repeated three times and the above is a representative experiment (n=3).

5.4 DISCUSSION

Through evaluation of the Defb14 inspired peptides, this study has shown the following:

- That fragments associated closely with the N-terminus, Dip 1, 4 and 5 display potent antimicrobial activity comparable with that of the full parent Defb14 peptide sequence
- That peptides associated with the C-terminus, Dip 2 and 3, display no antibacterial activity at the concentrations tested
- That Defb14 inspired peptide fragments are not chemotactic with HEK293 CCR6 cells at the concentrations tested
- That Dip 1, 2 and 3 display similar haemolytic profiles despite differences in hydrophobicity
- That Dip 1 displays similar salt insensitive antimicrobial activity to that of the parent Defb14 regarding *P.aeruginosa*.

5.4.1 Antimicrobial analysis of Defb14 inspired peptides

In order to dissect the antimicrobial activity of Defb14 three initial fragments were created Dip 1, 2 and 3. Dip 1, the N-terminal 23mer has no cysteine residues and carries a net charge of +6 alongside the two C-terminal (22mer) fragments (Dip 2 & 3). In comparison with the parent molecule, Defb14 (+12), this charge is strongly reduced. Antimicrobial activity is only exhibited with Dip 1 the N-terminal fragment and not displayed with the C-terminal (Dip 2 & 3) peptide fragments. The hydrophobicities of the C-terminal fragments Dip 2 & 3 are very similar to that of the parent molecule (-11.54) (Table 5.1) however the hydrophobicity of Dip 1 is markedly increased (-1.62).

Since the N-terminal fragment Dip 1 is active against bacteria and the net charge of the peptide fragments do not vary the differences in hydrophobicity may propose a difference. Previous work on HBD3 (Kluver *et al.*, 2005) discusses that increasing the net charge and the hydrophobicity will increase the antimicrobial activity. Peptides with less cationic residues and moderate hydrophobicity are virtually inactive

whereas peptides with a high net charge and significant hydrophobic character are active. Regarding Dip 1 this exhibited potent killing at the concentrations tested. Concerning HBD3, the human orthologue of Defb14, various studies have been conducted regarding altering sequences and creating derivatives (Hoover *et al.*, 2003, Wu *et al.*, 2003, Kluver *et al.*, 2005, Krishnakumari *et al.*, 2006). The N-terminus sequence of HBD3 has been synthesised containing 17 residues and possessing a net charge of only +4 (Hoover *et al.*, 2003). This peptide has displayed antimicrobial activity against both Gram positive and negative organisms in agreement with Dip 1. Dip 1 display a net charge of +6 and contains five more hydrophobic residues than this peptide but is also six residues longer.

Further analysis of the Defb14 N-terminus initiated the synthesis of a further three defensin inspired peptides in hope to identify active fragments. Dip 4 (10mer), 5 (12mer) and 6 (10mer) were synthesised giving net charges of +3, +5 and +2 respectively. Antimicrobial activity was detected with both Dip 4 and 5 but not with Dip 6. Dip 7 was created without the first twelve amino acids and the last eleven amino acids of the sequence and displayed no activity against the pathogens tested. Dip 4 and Dip 5 possess activity and have a similar number of hydrophobic residues and net charge. Dip 6 displays no killing at the concentrations tested but does remain furthest from the start of the N-terminus sequence. This peptide also contains double the hydrophobic residues (6) and carries a low net charge (+2). Previous studies have shown when using N-terminal deletion mutants that the initial seven residues are important for activity against gram positive organisms (Hoover *et al.*, 2003). Dip 4 and 5 possess activity against *S.aureus* but display higher values than those required for Dip 1. Dip 5 must be brought to attention as this sequence has lost the first five residues from the N-terminus but yet still displays activity against Gram positive organisms. Dip 6 has lost the first thirteen residues from the N-terminus and this may be important in the lack of activity at the concentrations tested.

An α-helix is often present in the N-terminal stretch of full length oxidised β-defensins (HBD1-3). N-terminal regions of defensins are often more aliphatic, and since helices are a frequent motif in antimicrobial peptides this has been postulated to be the

region of the peptide which directs membrane insertion and disruption. It is also interesting to note that Dip1, 6 and 7 contains a sequence of eight amino acids (GGRAAVLN) which are identical to that of the XT4 peptide fragment of an antimicrobial peptide isolated from the skin secretions of the diploid frog (Ali *et al* 2001). However this 22mer peptide is only active against Gram negative bacteria where Dip1 is active against Gram positive and negative organisms (charge +6, hydrophobicity -1.62) and Dip 6 and 7 display no activity at the concentrations tested (+2, -2.08; +2, -7.64).

The C-terminus of HBD3 has also been studied extensively. Derivatives of the last ten residues of the peptide sequence have been created. This peptide carries a net charge of +6 and consists of ten residues none of which are hydrophobic. Antimicrobial activity was detected against Gram negative organisms but no activity was evident against Gram positive organisms at the concetrations tested (Hoover *et al.*, 2003). In contrast Dip 2 and Dip 3 focus on the C-terminus of Defb14 and display no antimicrobial action against any of the organisms tested. These two peptides contain five and four hydrophobic residues possess the same net charge of +6 and contain 22 residues. Dip 3 contains a cysteine residue in place of one of the alanine residues. This was added to allow the peptide to form a dimer although by mass spectrometry this was not the case as only the presence of a monomer was detected. Oxidation and folding will need to be performed to allow the formation of a dimer. No peptides in the Hoover study were similar in residue number, net charge and hydrophobicity to Dip 2 and Dip 3. This may account for the differences observed in activity although the closest in length was 20 residues with a net charge of +7. This gave no difference in antimicrobial activity to that of the peptide containing only ten of the residues.

5.4.2 Antimicrobial activity in the presence of salt and serum concentrations

The antimicrobial activity of Dip 1, 4 and 5 were evaluated at various salt concentrations. The defensin inspired peptides remained unaffected when tested in this range against *P.aeruginosa* PAO1. No other organisms were tested. Studies involving derivatives of HBD3 particularly the C-terminus (Hoover *et al.*, 2003), show that

antimicrobial activity is completely diminished at 150 mM NaCl against *E.coli*. The N-terminal fragment of HBD3 displayed no measurable antimicrobial activity at the concentrations tested at 150 mM against both *E.coli* and *S.aureus*.

Dip 1 at 50 μg/mL was tested in the presence of 0.1%, 1% and 10% human serum. Dip displayed killing activity at the serum concentrations tested although at 10 % complete killing was not observed. In the same Hoover study the N and C-terminal fragments were tested for activity in the presence of 1 mM Mg and 2 mM Ca. Yet again no detectable activity was found against *E.coli* and *S.aureus* for N-terminal fragment. The C-terminal fragment was tested against just *E.coli* in the presence of the same 1 mM Mg and 2 mM Ca and displayed no measurable activity within the concentrations tested. HBD3 derivatives of the N-terminus where in one peptide the cysteines are replaced with alanine residues and in the other the cysteines are replaced with tryptophan residues (Kluver *et al.*, 2006) were tested for activity in the presence of Mueller-Hinton broth. Antimicrobial activity appeared to diminish in the presence of a quarter Mueller-Hinton broth. Both the N-terminal peptides contained 17 residues with the initial five residues of HBD3 removed completely. The C-terminal peptides in the same study contained 27 residues and displayed the same diminished activity in broth containing a quarter Mueller-Hinton.

5.4.3 Chemoattractant analysis

Chemotactic activity was evaluated with the initial three defensin inspired peptides. HEK293 cells transfected with CCR6 were used in the assay against Dip 1, 2 and 3. No migration was observed using the concentrations tested against this cell type. The disulfide connectivities have previously been reported to be essential for the receptor binding and interaction (Wu *et al.*, 2003). In this study the full length HBD3 was tested whereby the cysteine residues were replaced with aminobutyric acid, giving a completely linear peptide devoid of all disulfides. This peptide displayed no chemoattractant properties against the cell types tested. Monocytes and HEK293 CCR6 cells were inactive at concentrations less than 10000 ng/ml. Regarding the defensin inspired peptides no other cell types were tested and this should be evaluated before

completely concluding that no chemoattractant properties were observed. In the previous chapter Defb14 1cys was tested for chemoattractant properties. This peptide contained only one cysteine residue from the six cysteine backbone. This peptide displayed chemoattractant properties with monocytes at 1000 ng/ml and HEK293 CCR6 at 100 ng/ml. Importantly this peptide does not contain the proposed Asp^4-Leu^9 motif found in HBD2, which resembles the Asp^5-Leu^8 motif of CCL20/MIP-3α considered responsible for specific interaction with CCR6 (Hoover *et al.*, 2002). This motif is also not displayed in Dip 1, 2 and 3 as the cysteine residues are all replaced with alanine residues.

5.4.4 Haemolytic properties

The haemolytic profiles of Dip 1, 2 and 3 were evaluated. All three defensin inspired peptides gave similar haemolytic profiles which were also consistant with those of the full peptide and the one cysteine variant in the previous chapter. The net charge of Dip 1, 2 and 3 are all + 6 with the hydrophobicity of Dip 2 and 3 very similar (Table 5.1). Dip 1 gave an increased hydrophobicity but all three peptides displayed similar haemolysis and no cytotoxic properties were observed below 250 μg/ml. The haemolytic activity of HBD3 has been studied extensively (Kluver *et al.*, 2005). This research infers that a greater hydrophobicity encourages cytotoxic effects. Peptides with moderate hydrophobicity are relatively inactive and peptides displaying high net charge and hydrophobic properties are cytotoxic. This study does not display the haemolytic results of the N-terminal and C-terminal peptides created whereby the cysteine residues are replace with both alanines and tryptophan residues.

In summary, these results highlight that Dip 1, 4 and 5 which are based on the N-terminus sequence of Defb14 display potent antimicrobial properties similar to those of the parent Defb14 and Defb14 1 cys. In addition all the defensin inspired peptides displayed no chemoattractant properties against cells expressing CCR6. This in turn is an important aspect to consider in the development of defensin inspired peptides for potential therapeutic use.

CHAPTER 6:

SUMMARY AND FUTURE STUDIES

6.1 SUMMARY

A major hurdle for established antimicrobials has been the recent inability to combat multiple drug resistant organisms. Antimicrobials are currently failing to control or eliminate resistant infections. Addressing this problem would greatly improve the life span and decrease costs of treatment. New targets and classes of antimicrobials are required to help alleviate the problem.

Antimicrobial peptides are important components of the immune system's ability to fight disease. They are widely distributed throughout the animal and plant kingdom and rapidly inhibit infection. In numerous cases these peptides provide additional roles acting upon the immune system to coordinate a protective response. The β-defensins display antimicrobial properties and chemoattractant roles within host defence.

In this thesis murine β-defensins and defensins inspired peptides were analysed for functional significance. Initial studies focused on a novel five cysteine defensin related peptide termed Defr1. The work in this thesis showed that Defr1 displayed potent antimicrobial activity against a variety of pathogens including multi-resistant isolates and 5YC, the six cysteine analogue, displayed poorer activity. However the MBC values were not as low as established antimicrobials and the isolates were not tested in the presence of varying salt conditions. Defr1 has been shown to be a covalent dimer containing an intermolecular disulfide bond and is a mixture of dimeric isoforms, varying in intra and inter-molecular disulfide connectivities (Campopiano *et al.*, 2004). 5YC forms a non-covalently bound dimer displaying the characteristic β-defensin connectivity. In an attempt to study the isoforms of Defr1, HPLC fractions were collected at various time points and analysed by mass spectrometry, gel electrophoresis and antimicrobial function. All fractions revealed similar profiles antimicrobially and consisted of a mixture of isoforms like that of the original Defr1. Antimicrobial activity was also abolished when HPLC fractions were treated with DTT. To confirm the activity of a single dimeric species Defr1 1cys was created. This thesis showed that Defr1 1cys has a similar antimicrobial profile to that of the original Defr1. Defr1 1cys was also a dimer but was a single species and again upon reduction the antimicrobial activity was diminished like that of the mixed isoform Defr1. Chemoattractant analysis

of Defr1, 5YC and Defr1 1cys discovered that all three displayed migratory properties with $CD4^+$ T cells. Interestingly this thesis showed that not all cysteine residues were required for this to be displayed although all were dimeric. This thesis also reported that all three peptides did not display migratory properties with HEK293 cells transfected with CCR6, the proposed receptor for the β-defensins. This is currently the first report of a β-defensin not using CCR6 although additional receptors have been suggested for HBD3 (Wu *et al.*, 2003).

This thesis is the first functional report regarding Defb14 the murine orthologue of human β-defensin 3. Defb14 was synthesised and displayed potent antimicrobial properties, similar to those experienced with the orthologue HBD3. In addition this activity, in contrast to Defr1 was not diminished in the presence of DTT. Defb14 was shown to be monomeric and a mixture of different disulfide connectivities therefore a one cysteine dimeric variant was synthesised. This peptide displayed antimicrobial properties similar to that of the original monomeric Defb14. Chemotactic analysis revealed that Defb14 and Defb14 1cys displayed migratory properties for $CD4^+$ T cells and HEK293 CCR6 transfected cells again showing that the six cysteine residues are not required for chemotactic activity in vitro. Monocytes were also attracted to Defb14 and these do not express a functional CCR6. This is comparable with experiments done with HBD3 where similar properties are observed with chemotaxis.

The Defb14 inspired peptides (Dips) provided an insight into drug design and the regions of the peptide required to produce activity. Peptides based on the C-terminus sequence displayed no antimicrobial properties but peptides based on the sequence of the N-terminus did. Interestingly smaller N-terminal fractions continued to provide antimicrobial activity. These peptide fragments have highlighted regions of activity but do not confirm that the N-terminus is solely required for activity in vivo. Further peptides will have to be created to elucidate the areas attributed to the effect. Regarding chemotaxis, the Dips do not display migratory properties against the HEK293 cells expressing CCR6 thus the antimicrobial and chemoattractant properties can be separated. This provides further validation that the β-defensins can provide a scaffold for further drug design.

6.2 FUTURE STUDIES

Defr1

1. Since Defr1 is a variant allele of Defb8 it would be of interest to synthesise Defb8 and study the antimicrobial profile and its ability to perform under reduced conditions.

2. Furthermore studies of the chemoattractant properties of Defb8 would be advantageous and interesting to confirm if this peptide uses the same unknown receptor as that of Defr1 or a different receptor.

3. Relating to dimeric structure being important for antimicrobial activity with Defr1, 5YC and Defr1 1cys, the analysis of a monomeric preparation of Defr1 1cys would have great interest particularly in the area of chemotaxis.

Defb14

4. It is unclear from the studies conducted in this thesis what the additional receptor(s) Defb14 utilises in order to chemoattract monocytes. Identification of this receptor would be helpful in understanding its role within the immune system particularly if this is the same additional receptor used by HBD3.

5. Chemotaxis was not observed with the defensin inspired peptides nor with Defb14 without any cysteine residues. Ideally forcing Dip3 to make a dimer would be interesting to confirm if structural aspects are required for interaction with CCR6.

6. Creating Defb14 1cys with biotin attached would provide a useful tool to fish out the receptor and for membrane acting studies. Analysis of bioactivity would have to be conducted first.

7. Further mutations in the sequence of the N-terminus of the defensin inspired peptides could potentially reveal the important residues required for antimicrobial action.

8. Changing the sequence to incorporate residues important for salt resistance, increasing potency and reducing toxicity would contribute to the drug development process.

9. Developing a murine knockout of the β-defensin cluster would be beneficial in determining the defensin functions in vivo. Using the Lox P Cre recombinase system the cluster could be removed although defensins are present at other loci and may compensate for this initial loss.

10. Perhaps a more elegant system would be firstly to identify the enzyme required for processing the β-defensin and inactivate this, like the matrilysin studies for the α-defensins. This strategy would inactivate the defensin genes.

11. Isolating Defb14 and Defr1 from their natural sources rather than analysing the synthetic peptides would confirm if the disulfide connectivities are mixed or if the peptides retain the native β-defensin conformation.

12. Co-crystalise CCR6 and a β-defensin would demonstrate the exact residues and bonds that interact. Removing these from the peptide could potentially allow chemotaxis to be removed.

13. Over expression of Defb14 would determine the relevance of this molecule in vivo.

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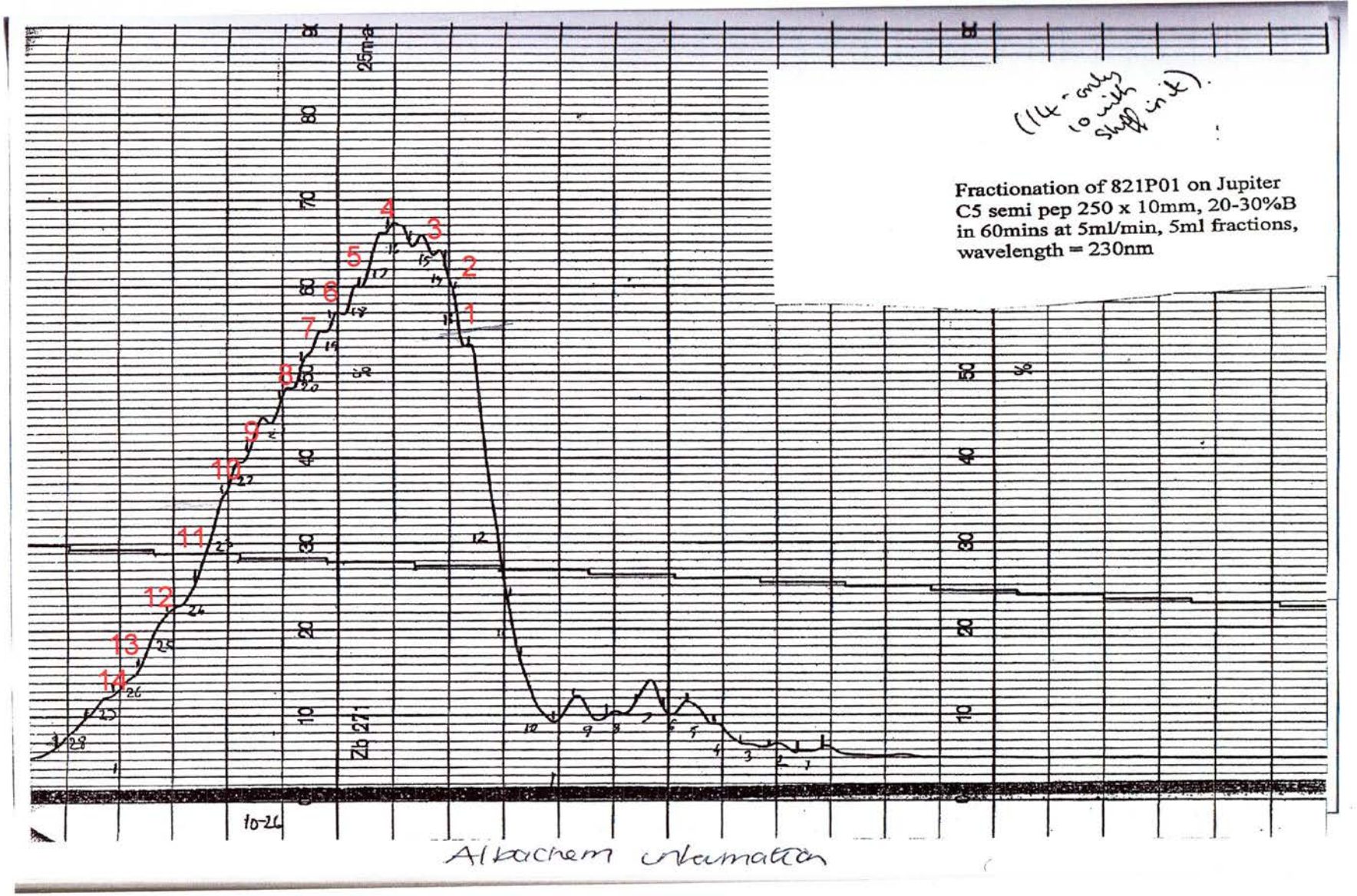
APPENDIX



Appendix 1 : Albachem HPLC trace (fractions 1-14 labelled)